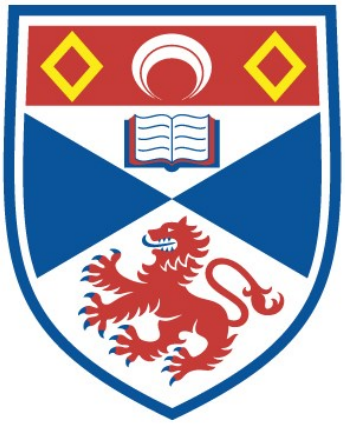
STUDIES ON THE SYNTHESIS AND TURNOVER OF ARGININE AND LEUCINE: TRNA LIGASES IN CULTURED TOBACCO CELLS

Nigel Robert Gore

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1975

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By NIGEL ROBERT GORE

ABSTRACT

A technique was developed for assaying amino acid: tRNA ligases extracted from tobacco XD cells grown in chemically defined medium (M-1D). The technique was based on the physiological enzymic reaction in which amino acid is aminoacylated to tRNA. tRNA was obtained from tobacco XD cells using a phenol extraction procedure. For two enzymes, arginine: tRNA ligase and leucine: tRNA ligase, assay conditions were optimised. Both enzymes had similar Km values for their cognate amino acids; were found to be unstable when stored at -10' and their activity was inhibited by ammonium sulphate and caesium chloride.

During growth of tobacco XD cells, these two enzymes increased in activity. Amino acids appeared not to be involved in their regulation and attempts to perturb *in vivo* levels of aminoacyl-tRNA by use of amino acid analogues were unsuccessful. The use of the density labelling technique, which allows a distinction between pre-existing enzyme molecules and those that are newly synthesised, indicated that in M-1D both arginine; and leucine: tRNA ligases were synthesised *de novo*. Leucine: tRNA ligase was also degraded and therefore turned over as it increased in activity. The density labelling data did not allow a similar conclusion for arginine: tRNA ligase. During cell growth in nitrateless M-1D, there was no increase in the activity of arginine: and leucine: tRNA ligases, but both anzymes were found to be synthesised *de novo*. It was concluded, therefore, that they were both degraded and so turned over in nitrateless M-1D. Arginine: and leucine: tRNA ligases appeared to be synthesised from different amino acid pre-cursor pools and DEAE cellulose chromatography of enzyme extracts revealed the presence of three ligase species cognate for arginine but only two species cognate for leucine. The species cognate for arginine were in approximately equal proportions whereas one of the species cognate for leucine accounted for 80% of the total enzyme activity. The possibility that these multiple enzymic species might be responsible for the inability to demonstrate degradation of the arginine anzyme in M-1D was discussed. An accurate determination of the turnover rates of these/

these two enzymes could not be obtained due to the effects of re-cycling of total cell protein, but a comparison of turnover rates was attempted. The possible mode of regulation of these enzymes was discussed in relation to our observations and to those found in other systems.

STUDIES ON THE SYNTHESIS AND TURNOVER OF ARGININE: AND LEUCINE: tRNA LIGASES IN CULTURED TOBACCO CELLS

by

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A thesis submitted to the University of St. Andrews in application for the degree of Doctor of Philosophy

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November 1975

DECLARATION

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition, and that no part of it has been presented previously for a higher degree.

The research was carried out in the Department of Biochemistry of the University of St. Andrews under the direction of Dr. J. L. Wray.

CERTIFICATE

I hereby declare that Nigel Robert Gore has spent nine terms in research work under my direction and that he has fulfilled the conditions of Ordinance No. 16 (St. Andrews), and that he is qualified to submit this thesis for the degree of Doctor of Philosophy.

ACADEMIC RECORD

I matriculated at the University of St. Andrews in October 1968 and graduated with the degree Bachelor of Science, Upper Second Class Honours in Biochemistry, in June 1972.

In October 1972, I matriculated as a research student at the University of St. Andrews.

ACKNOWLEDGMENTS

I should like to thank my supervisor, Dr. J.L. Wray for his encouragement, advice and criticism throughout this work; the Science Research Council for their award of a studentship; Mr. R.E. Brice and Mr. P.D. Talbot for their technical assistance and Miss A. Ratnage for the typing.

C O N T E N T S

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SUMMARY

A technique was developed for assaying amino acid: tRNA ligases extracted from tobacco XD cells grown in chemically defined medium (M-1D). The technique was based on the physiological enzymic reaction in which amino acid is aminoacylated to tRNA. tRNA was obtained from tobacco XD cells using a phenol extraction procedure. For two enzymes, arginine: tRNA ligase and leucine: tRNA ligase, assay conditions were optimised. Both enzymes had similar Km values for their cognate amino acids; were found to be unstable when stored at -10° and their activity was inhibited by ammonium sulphate and caesium chloride.

During growth of tobacco XD cells, these two enzymes increased in activity. Amino acids appeared not to be involved in their regulation and attempts to perturb *in vivo* levels of aminoacyl-tRNA by use of amino acid analogues were unsuccessful. The use of the density labelling technique, which allows a distinction between pre-existing enzyme molecules and those that are newly synthesised, indicated that in M-1D both arginine; and leucine: tRNA ligases were synthesised *de novo*. Leucine: tRNA ligase was also degraded and therefore turned over as it increased in activity. The density labelling data did not allow a similar conclusion for arginine: tRNA ligase. During cell growth in nitrateless M-1D, there was no increase in the activity of arginine: and leucine: tRNA ligases, but both enzymes were found to be synthesised *de novo*. It was concluded, therefore, that they were both degraded and so turned over in nitrateless M-1D. Arginine: and leucine: tRNA ligases appeared to be synthesised from different amino acid pre-cursor pools and DEAE cellulose chromatography of enzyme extracts revealed the presence of three ligase species cognate for arginine

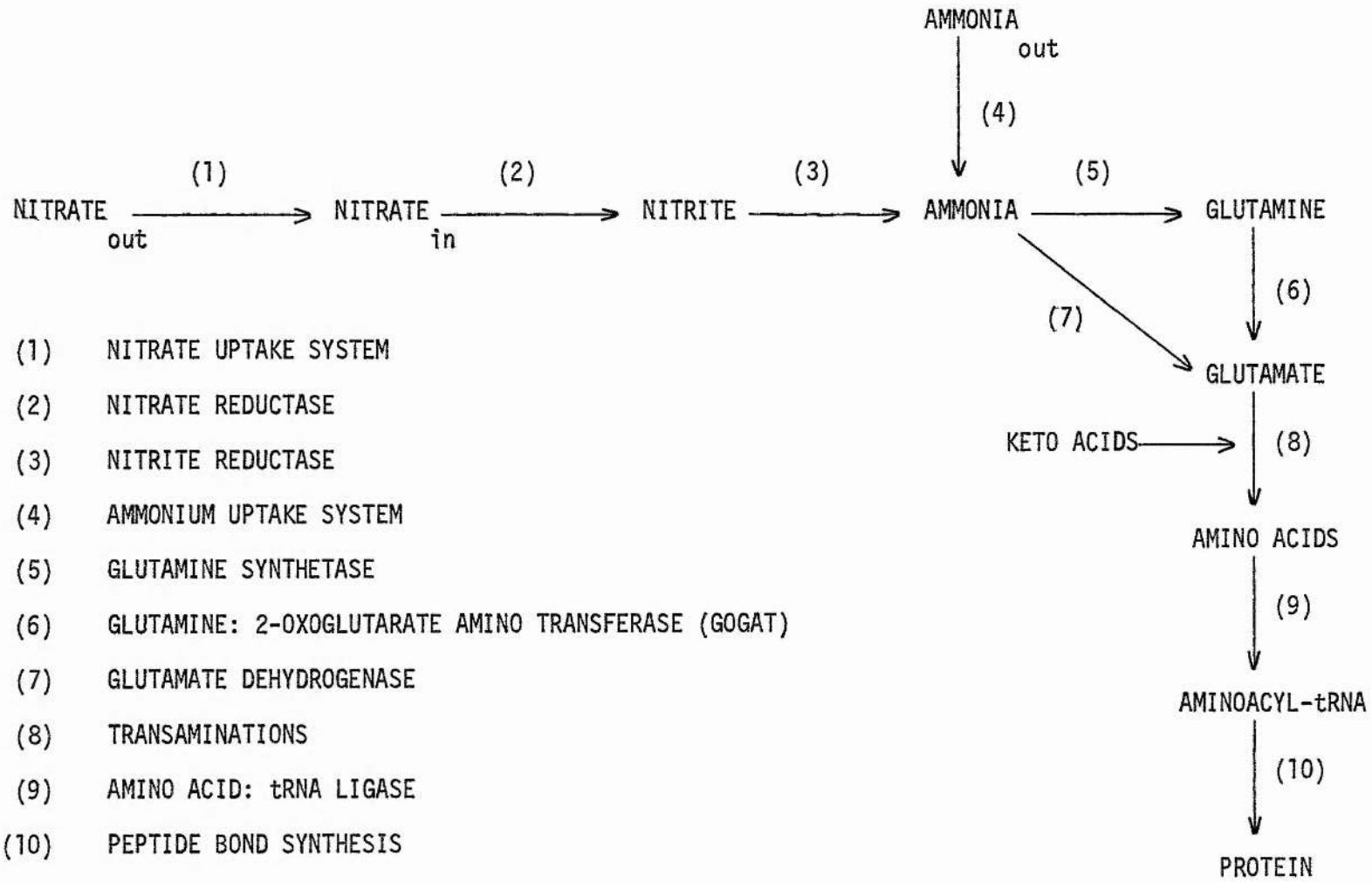
but only two species cognate for leucine. The species cognate for arginine were in approximately equal proportions whereas one of the species cognate for leucine accounted for 80% of the total enzyme activity. The possibility that these multiple enzymic species might be responsible for the inability to demonstrate degradation of the arginine enzyme in M-1D was discussed. An accurate determination of the turnover rates of these two enzymes could not be obtained due to the effects of re-cycling of total cell protein, but a comparison of turnover rates was attempted. The possible mode of regulation of these enzymes was discussed in relation to our observations and to those found in other systems.

INTRODUCTION

Nitrogen is an important element cycling through living organisms and is an essential requirement for the synthesis of proteins and nucleic acids. Molecular nitrogen, however, although occurring in large quantities in the atmosphere can only be reduced directly by blue green algae and nitrogen fixing bacteria such as Rhizobium, Azotobacter and the Actinomycetes (Dilworth, 1974; Fogg, 1974). Other organisms must obtain their nitrogen in some combined form such as nitrate, nitrite, ammonia or amino acids.

As far as plants are concerned nitrate is by far the most important source of inorganic nitrogen because although ammonia is freely taken up and is often provided in fertilisers, the microbial oxidation of ammonia to nitrate is rapid and nitrogen fixation by symbiosis is usually only maximum when combined nitrogen is limiting (Stewart, 1966). The mechanism by which external soil nitrate is converted to plant protein may be considered to consist of three main areas, viz. (1) the nitrate assimilation pathway, (2) amino acid biosynthesis, and (3) protein synthesis (Fig. 1).

The nitrate assimilation pathway converts nitrate in the environment to ammonia via a system including a nitrate uptake mechanism and two enzymes, nitrate reductase and nitrite reductase, thereby making nitrogen available for a variety of biosyntheses, the most important quantitatively being protein synthesis (Kessler, 1964; Beevers & Hageman, 1969). However, little is known about the uptake system in comparison to the abundant information available about the enzymic steps (Hewitt, 1975). Nitrate reductase is a molybdoflavoprotein and is located in the cytoplasm (Ritenour et al., 1967; Grant et al., 1970; Dalling et al., 1972). It uses NADH as electron donor (Beevers et al., 1964) derived largely from the oxidation of photosynthetically produced 3-phosphoglyceraldehyde by triose phosphate dehydrogenase (Klepper et al., 1971). Nitrite reductase is an iron-sulphur protein,



(1) NITRATE UPTAKE SYSTEM

(2) NITRATE REDUCTASE

(3) NITRITE REDUCTASE

(4) AMMONIUM UPTAKE SYSTEM

(5) GLUTAMINE SYNTHETASE

(6) GLUTAMINE: 2-OXOGLUTARATE AMINO TRANSFERASE (GOGAT)

(7) GLUTAMATE DEHYDROGENASE

(8) TRANSAMINATIONS

(9) AMINO ACID: tRNA LIGASE

(10) PEPTIDE BOND SYNTHESIS

Fig. 1 Conversion of soil nitrate to plant protein

located in the chloroplast (Losada et al., 1963; Ritenour et al., 1967; Magalhaes et al., 1974) and appears to use ferredoxin as the physiological electron donor (Losada et al., 1963; Neyra & Hageman, 1974).

A number of investigators have established that the chloroplasts are a major site for both amino nitrogen synthesis (Bassham & Kirk, 1964; Magalhaes et al., 1974) and amino acid biosynthesis (Tsukamoto, 1970; Kirk & Leech, 1972) in green plants. It has been proposed that ammonia is initially assimilated into the carbon chain of 2-oxoglutarate via the action of glutamate dehydrogenase. However, although this enzyme has been identified in chloroplasts (Leech & Kirk, 1968), it is low in activity and possesses a high Km for ammonia (Lea & Thurman, 1972) and thus a low capacity for producing glutamate.

Following the recent discovery in yeast (Brown et al., 1973) and bacteria (Brown et al., 1974, Brown & Dilworth, 1975) of an enzyme which transfers the amide group of glutamine to 2-oxoglutarate, Lea & Miflin (1974) have reported the presence of a similar enzyme in the chloroplasts of pea leaves which is ferredoxin dependent. This enzyme is called glutamine:2-oxoglutarate amino transferase (GOGAT) and in combination with glutamine synthetase, an enzyme which has a high activity and a low Km for ammonia (Miflin, 1974), ammonia can be incorporated into the α-amino group of glutamate in the chloroplast. It is proposed that the glutamine synthetase/GOGAT system plays a major role in α-amino nitrogen synthesis at low ammonia concentrations, whereas at high concentrations α-amino nitrogen synthesis is via glutamate dehydrogenase.

Once assimilated into the amino group of glutamate, other amino acids can be synthesised via amino transferase reactions with keto acids (Gamborg, 1965). Kirk & Leech (1972) suggest that for chloroplasts isolated from Vicia faba, glutamate is converted specifically

to alanine and aspartate and that it is by amino transfer from these two latter amino acids that the other amino acids are synthesised. Generally it is thought that the pathways of amino acid biosynthesis in plants closely resemble those found in bacteria and the present situation has recently been reviewed by Miflin (1973).

The most complex part of nitrogen metabolism is the polymerisation of amino acids to proteins. This has been recently reviewed (Boulter et al., 1972; Zalik & Jones, 1973) and the sequence of events leading to protein can be summarised in the following steps:-

(1) activation of amino acids and formation of aminoacyl-tRNA molecules.

(2) formation of a competent complex between ribosomes and mRNA.

(3) binding of aminoacyl-tRNA to this complex.

(4) initiation of chain growth and formation of peptide bonds in the complex.

(5) release of completed protein chains from the complex.

The enzymes with which we are concerned in this thesis catalyse the first step in this process, that is, the two stage transfer of amino acid to tRNA which can be represented by the following equations:-

(1) Amino acid + ATP + Enzyme $\rightleftharpoons$ /Aminoacyl-AMP-Enzyme/ + PPi

(2) /Aminoacyl-AMP-Enzyme/ + tRNA $\longrightarrow$ Aminoacyl-tRNA + AMP

The first step involves the activation of the amino acid carboxyl group and the second, the transfer of amino acid to tRNA. The enzymes catalysing these reactions are called amino acid:tRNA ligases (E.C.6.1.1.), aminoacyl-tRNA synthetases or amino acid activating enzymes and are found in all living cells. There appears to be at least one enzyme cognate for each amino acid and to ensure strict fidelity in the translation of the genetic code, the overall reaction must exhibit exclusive specificity both for cognate amino acid and tRNA.

Hoagland in 1955 was the first to demonstrate the existence of these enzymes when he found that a preparation from the soluble protein of rat liver catalysed an amino acid dependent exchange of $[^{32}P]$-pyrophosphate with ATP. Similar observations were made in E. coli extracts (DeMoss and Novelli, 1956) and in the following year Webster (1957) found that ribonucleoprotein particles isolated from pea seedlings also had this ability. In 1959 ATP-pyrophosphate exchange was observed in pig liver and yeast (Webster, 1959) and it was therefore becoming evident that these enzymes were present in a large number of tissues. Prior to 1960 plant ligases had only been identified in pea (Davis & Novelli, 1958; Clark, 1958; Webster, 1959), spinach (Clark, 1958; Marcus, 1959; Bové & Raacke, 1959) and carrot root (Davis & Novelli, 1958) but have subsequently been discovered in a large number of plant tissues (Lea & Norris, 1972). In many cases individual enzymes have been partially purified and their properties found to be similar to their animal and bacterial counterparts. A more detailed account of the distribution and characterisation of arginine: and leucine: tRNA ligases, the two enzymes with which we are specifically concerned in this thesis is given in Table 1.

In a number of plants, the presence of multiple amino acid: tRNA ligases cognate for a particular amino acid have been observed. For example two enzymes cognate for tyrosine have been identified in pea roots (Cowles & Key, 1972, 1973) and Kanabus & Cherry (1971) have isolated three enzymes cognate for leucine in soybean cotyledons but only two in the hypocotyls. Multiple forms of this enzyme have also been reported in Phaseolus vulgaris (Williams & Williams, 1970). This is in contrast to the situation in micro-organisms where usually only one enzyme species is observed, although two enzymes cognate for arginine have been described in E. coli (Yem & Williams, 1973). The presence of multiple enzymes in plants is not surprising in view of

Table 1. Detection of arginine: and leucine: tRNA ligases in some higher plants

Plant source	Amino acid: tRNA ligase		Partially purified		Assay method		Author
	arginine	leucine	arginine	leucine	aminoacylation of tRNA	ATP-pyrophosphate exchange	
Spinach (Spinacea oleracea)	+	+	-	-	-	+	Clark (1958)
	-	+	-	-	-	+	Marcus (1959)
	-	+	-	-	-	+	Bové & Raacke (1959)
Pea (Pisum sativium)	+	+	-	-	-	+	Davis & Novelli (1958)
	+	+	-	-	-	+	Henshall & Goodwin (1964)
	+	-	+*	-	+	+	Aliev & Filippovich (1968)
	+	+	-	-	+	-	Scott & Morris (1969)
	+	+	+	-	+	-	Cowles & Key (1973)
	-	+	-	+*	+	-	Wright et al. (1974)

Table 1 contd.

Plant source	Amino acid: tRNA ligase		Partially purified		Assay method		Author
	arginine	leucine	arginine	leucine	aminoacylation of tRNA	ATP-pyrophosphate exchange	
Goats rue (Galega officinalis)	+	+	-	-	-	+	Moustafa & Proctor (1962)
Wheat (Triticum aestivum)	+	+	-	-	+	+	Moustafa & Lyttelton(1963)
	+	+	-	-	-	+	Norris et al. (1973)
Tomato (Sutton's 'best of all')	+	+	-	-	hydroxamate formation		Attwood & Cocking (1965)
Field bean (Vicia faba)	+	+	-	-	-	+	Hinde et al. (1966)
Tobacco (Nicotiana tabacum)	-	+	-	-	-	+	Anderson & Rowan (1966)
	+	+	-	-	+	-	Guderian et al. (1972)
	+	+	-	-	-	+	Wray et al. (1974)
	+	+	-	-	+	-	Cornelis & de Patoul (1975)

Table 1 contd.

Plant source	Amino acid: tRNA ligase		Partially purified		Assay method		Author
	arginine	leucine	arginine	leucine	aminoacylation of tRNA	ATP-pyrophosphate exchange	
Tobacco (Nicotiana rustica)	-	+	-	-	+	-	Nathan & Richmond (1974)
Lupin (Lupinus luteus)	-	+	-	+	+	+	Legocki & Pawelkiewicz (1967)
Jack bean (Canavalia ensiformis)	+	-	-	-	-	+	Fowden & Frankton (1968)
Soybean (Glycine max)	-	+	-	-	+	-	Anderson & Cherry (1969)
	-	+	-	-	+	-	Bick et al. (1970)
	-	+	-	-	+	-	Bick & Strehler (1971)
	-	+	-	+*	+	-	Kanabus & Cherry (1971)

Table 1 contd.

Plant source	Amino acid: tRNA ligase		Partially purified		Assay method		Author
	arginine	leucine	arginine	leucine	aminoacylation of tRNA	ATP-pyrophosphate exchange	
French bean (Phaseolus vulgaris)	+	+	-	-	-	+	Anderson & Fowden (1969)
	+	+	-	-	+	+	Burkard et al. (1970)
	-	+	-	+*	+	-	Guillemaut et al. (1975)
Horse chestnut (Aesculus hippocastanum)	-	+	-	+	-	+	Anderson & Fowden (1970)

* Fractionation of multiple ligase species

the high degree of compartmentation compared with micro-organisms and the well documented fact that both chloroplasts and mitochondria are able to synthesise part of their protein complement on their own ribosomes (Boulter et al., 1972). Chloroplast enzymes have been located in spinach (Bové & Raacke, 1959), pea seedlings (Sissakian et al., 1965; Aliev & Filippovich, 1968), Phaseolus vulgaris (Burkard et al., 1970) and tobacco leaf (Francki et al., 1965; Guderian et al., 1972). Mitochondrial enzymes have been reported in carrot root (Davis & Novelli, 1958), mung bean (Peterson, 1964), tobacco leaf (Guderian et al., 1972) and Phaseolus vulgaris (Guillemaut et al., 1975). In addition there have been a number of reports of protein synthesis and amino acid: tRNA ligase activity in nuclei (Birnsteil et al., 1962; Flamm et al., 1963; Chipchase & Birnsteil, 1963), protein bodies (Morton & Raison, 1964; Morton et al., 1964a b), and ribosomes but many of these reports can be accounted for by bacterial contamination (Wilson, 1966; Wheeler & Boulter, 1966).

Although there is considerable variation in the levels of individual amino acid: tRNA ligases in plant tissues it is generally found that the enzymes cognate for isoleucine, leucine and valine are of high activity while those for arginine, aspartate, glutamate and glutamine are usually low. The remaining enzymes tend to be more variable (Lea & Norris, 1972). These differences may reflect the true in vivo situation or could arise from the experimental manipulation either of the tissue during extraction or during subsequent assay. In some tissues the low activity found for the glutamate (Lea & Fowden, 1972) and glycine (Niyomporn et al., 1968) enzymes appear to result from their instability during extraction. Both the enzymes for arginine (Mitra & Mehler, 1966; Mitra & Smith, 1969) and glutamine (Ravel et al., 1965) may have a requirement for tRNA in the activation step, whereas the low activity of the enzyme cognate for alanine seen in a

number of studies (Anderson & Fowden, 1969; Wray et al., 1974) is probably a consequence of the very high Km for alanine in the ATP-pyrophosphate exchange assay (Attwood & Cocking, 1965).

Many investigators have demonstrated changes in activity in tissue that is undergoing active growth and development. Examples of such changes have been reported in germinating pea (Henshall & Goodwin, 1964), bean (Anderson & Fowden, 1969) and wheat (Norris et al., 1973) seedlings; in developing bean (Hinde et al., 1966) and pea (Cowles & Key, 1973) roots; during growth of tobacco tissue culture (Wray et al., 1974) and in such diverse tissues as Streptococcus thermophilus (Nurmikko et al., 1965) and sea urchin (Spadafora et al., 1973). Changes in ligase activity have also been reported during senescence of soybean cotyledons (Bick & Strehler, 1971) and tobacco leaves (Nathan & Richmond, 1974), suggesting that these enzymes may play an important role in cellular differentiation.

In addition there are multiple iso-accepting tRNA species for an amino acid, some of which may be organelle specific (Burkard et al., 1970; Guderian et al., 1972; Guillemaut et al., 1975) and whose levels may change during growth and development (Vold & Sypherd, 1968; Anderson & Cherry, 1969; Bick et al., 1970). It is possible therefore that cellular differentiation may be modulated not only by changes in ligase levels but also by changes in their ability to aminoacylate different tRNA species. The observation that multiple enzymic forms also vary in amount during growth and development (Cowles & Key, 1973) suggests the involvement of an intricate and complex regulatory mechanism.

However, amino acid: tRNA ligases are not the only enzymes which show characteristic changes during development. Increases in enzyme activity are common during germination and seedling development. For example, malate synthetase and isocitrate lyase have been found by

a number of investigators to increase during germination in both the endosperms and cotyledons of fatty seedlings (Carpenter & Beevers, 1959; Yamamoto & Beevers, 1960; Lee et al., 1964; Marcus & Feeley, 1964). There are also numerous reports of other cotyledonary enzymes which increase during germination (Prentice et al., 1967; Brown & Wray, 1968; Mandal & Biswas, 1970; King, 1970) and similar increases have been observed with several enzymes of the glycolytic and pentose phosphate pathways (Cornaggia, 1964). A more detailed account of changes in enzyme levels during growth and development and also in response to various environmental factors can be found in reviews by Filner et al. (1969) and Marcus (1971).

In recent years much attention has been focussed on the question of whether these changes in activity are the result of protein synthesis, activation of a pre-formed protein precursor or removal or destruction of an inhibitor. Many of the earlier attempts to resolve this question centred on the use of inhibitors of protein and RNA synthesis such as puromycin, cycloheximide and actinomycin D, based on the premise that if the inhibitor prevented the increase in enzyme activity then the enzyme was synthesised de novo. For example, treatment with such inhibitors prevents the rise of isocitrate lyase and malate synthetase in water melon cotyledons (Hock & Beevers, 1966) and the development of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in pea cotyledons (Brown & Wray, 1968). Similar observations have been made with a large number of enzymes in castor bean endosperm (Albergoni et al., 1964; Alberghina, 1964) and Henshall & Goodwin (1964) have reported that the amino acid analogue, p-fluorophenylalanine, prevents the rise in pea amino acid: tRNA ligases. However, the conclusion that de novo synthesis is responsible for these increases is not necessarily justified since processes involving activation may also depend on a functional protein synthetic apparatus. However, more sophisticated methods are now available to

investigate this problem:-

(1) Radioactive labelling:- This involves showing the incorporation of exogenously supplied labelled amino acids into enzyme protein, either by constant infusion or by pulse labelling of the tissue with isotope. To overcome the problems of compartmentation of metabolites found in plants (Oaks & Bidwell, 1970), modifications of these two methods have been made by a number of investigators (Hellebust & Bidwell, 1964; Oaks, 1965; Holleman & Key, 1967; Kemp & Sutton, 1971; Trewavas, 1972).

A more recent method shows the incorporation of ^3H into enzyme protein (Humphrey & Davies, 1975). This technique is based on the assumption that when tissue is incubated with ^3H_2O, ^3H rapidly equilibrates with hydrogen on the α-carbon atoms of most free amino acids, due to an exchange reaction catalysed by transaminases. When the tissue is transferred to water, the reverse reaction occurs and therefore only those amino acids which have been incorporated into enzyme protein will be labelled with ^3H. Isolation of labelled enzyme protein would therefore indicate de novo synthesis.

(2) Immunology:- This involves obtaining a quantitative precipitation of newly synthesised enzyme protein by antibody, and is often employed in combination with labelled amino acids. For example, de novo synthesis of ribulose 1,5-diphosphate carboxylase has been sucessfully demonstrated in barley leaves (Kleinkopf et al., 1970). However, these methods have been applied mainly to animal systems (Schimke & Doyle, 1970).

(3) Density labelling (Hu et al., 1962):- This technique involves generating a density difference between pre-existing and newly synthesised proteins by labelling with heavy stable isotope and then resolving the old and new proteins by isopycnic equilibrium

centrifugation in caesium chloride. We have employed this proceedure to look at the factors involved in the changes of arginine and leucine: tRNA ligases during growth of tobacco XD cells in suspension culture. Examples of the successful use of this method to demonstrate de novo synthesis of plant enzymes is shown in Table 2.

Another advantage of this technique is that it can be employed to show turnover of protein. The term 'turnover' means that at any particular point in time protein is undergoing both synthesis and degradation and therefore the net level of protein present is determined by both the rates of synthesis and degradation. In 1935, Borsook and Keighley proposed that all proteins in mammalian tissues were continually synthesised and degraded and with the advent of isotopic techniques, this was confirmed by Schoenheimer (1942) and subsequently by many investigators working with specific enzymes in animal systems (Schimke, 1969; Schimke & Doyle, 1970; Schimke, 1973).

Study of turnover in plant systems has considerably lagged behind that in animals and the majority of investigations have dealt with turnover to total protein (Huffaker & Peterson, 1974) rather than specific enzymes. However to date five plant enzymes have been shown to turn over (Table 2), while increasing in activity, emphasising that such changes could be due not only to protein synthesis but also to protein degradation. Generally, as far as animal systems are concerned, increases in enzyme activity are determined by the rate of enzyme synthesis.

In contrast, there is little turnover of proteins in micro-organisms (Mandelstam, 1958) with the exception of non dividing cells (Pine, 1966; Willetts, 1967). The situation is therefore different to that in animal systems, there being an 'all or none' effect, that is either synthesis or no synthesis of protein. Enzyme levels in the bacterial cell may therefore depend on two factors:- firstly the rate

Table 2. Enzymes which have been shown by density labelling to be synthesised de novo in response to various environmental parameters

Enzyme	Control factor	Tissue	Turnover demonstrated	Author
α-amylase	giberellic acid	barley seeds	-	Filner & Varner (1967)
isocitrate lyase	age	peanut cotyledons	-	Gientka-Rychter & Cherry (1968)
				Longo (1968)
malate synthetase	age	peanut cotyledons	-	Longo (1968)
peroxidase	age	barley seeds	-	Anstine et al. (1970)
nitrate reductase	nitrate	tobacco tissue culture	+	Zielke & Filner (1971)
catalase	age	maize scutella	+	Quail & Scandalios (1971)
acid phosphatase	age	pea cotyledons	-	Johnson et al. (1973)
invertase	wounding	carrot discs	-	Wray & Brice (1973)
ascorbate oxidase	phytochrome	mustard seedlings	+	Acton et al. (1974)
				Attridge (1974)
ribonuclease	phytochrome	lupin hypocotyls	+	Acton & Schopfer (1974)
malate dehydrogenase	age	maize scutella	+	Yang & Scandalios (1975)

of growth, since during a phase of rapid growth enzyme levels can be diluted out to a new steady state low level and secondly, whether genes are expressed, that is whether new protein is synthesised.

Regulation at the genetic level was proposed by Jacob & Monod in 1961. Their model envisages the synthesis of mRNA initiated near a specific region of DNA termed the operator. An operator is adjacent to and co-ordinates the expression of a group of structural genes which are responsible for specifying the primary structure of some or all the enzymes of a metabolic pathway. The group of structural genes together with its associated operator constitutes an operon. Also present in the DNA is a genetic element known as the regulator or 'i' gene which produces a repressor protein.

In repressible systems the end product (co-repressor) of a metabolic pathway allosterically interacts with the repressor protein and this product is then able to complex with the operator, preventing transcription of the structural genes. This is repression. If the intracellular concentration of the co-repressor falls, the repressor-co-repressor complex dissociates and the repressor reverts to a form that can no longer bind to the operator, thus permitting expression of the structural genes. This is derepression.

In inducible systems binding of the repressor protein to the operator region prevents transcription of the structural genes. An inducing substance allosterically interacts with the repressor protein, such that it can no longer bind to the operon, thus allowing the structural genes to be transcribed. This latter form of control is common to catabolic pathways whereas repression/derepression mechanisms are usually associated with anabolic pathways. However both these mechanisms allow a very precise control of enzyme levels in micro-organisms.

Although repression is a widespread phenomenon in micro-

organisms there is very little evidence for its existence in higher plants. In amino acid biosynthesis, although there are many reports of regulation by end product inhibition of enzyme levels (Miflin, 1973), attempts to demonstrate repression by increasing the intracellular level of a particular amino acid within a plant tissue have not been successful (Weber & Bock, 1968; Dougall, 1970; Widholm, 1971; Miflin & Cave, 1972; Chu & Widholm, 1972). The only well documented example of repression in higher plants is in the nitrate assimilation pathway of cultured cells of tobacco, where nitrate uptake (Heimer & Filner, 1971) and nitrate and nitrite reductases (Filner, 1966; Kelker & Filner, 1971) appear to be repressed by casein hydrolysate. Together with the evidence that these three activities are also induced by nitrate (Heimer & Filner, 1971; Kelker & Filner, 1971), it has been suggested that the nitrate assimilation pathway may constitute an operon similar to that found in micro-organisms (Anon, 1971). However the density labelling studies of Zielke & Filner (1971) clearly demonstrate that the control of nitrate reductase by casein hydrolysate is not via a true repression mechanism. Even when the enzyme is repressed by casein hydrolysate it is still turning over, that is still being synthesised and degraded, suggesting therefore, that the control of the enzyme level is not an 'all or none' process but a fine balance between synthesis and degradation. It would appear therefore that the regulation of plant enzyme levels is considerably different to that found in micro-organisms.

As far as plant amino acid: tRNA ligases are concerned, little is known about the mechanisms underlying the observed changes during growth and development or indeed how these changes are regulated at the molecular level. An involvement of amino acids has been suggested by Henshall & Goodwin (1964), since they correlated an increase in the free amino acid pool with an increase in ligase activity when pea

plants were treated with gibberellic acid. A similar observation has been made during growth of tobacco tissue culture (Wray et al., 1974) where an increase in free amino acid levels was observed just before maximum ligase activity. However Anderson and Rowan (1966a) showed that treatment of tobacco leaves with kinetin increased the specific activity of these enzymes whilst at the same time causing a decrease in the free amino acid pool.

An involvement of amino acids or some derivative has been implicated in other systems. In rat liver, protein depletion (starvation) leads to an increase in ligase levels (Mariani et al., 1963), suggesting that as the amino acid pool decreases, the level of these enzymes rises to increase the fraction of amino acid involved in protein synthesis. In yeast (Ehresmann et al., 1971) removal of valine from the culture medium of cells grown in the presence of amino acid causes a two fold increase in the specific activity of valine: tRNA ligase and the use of amino acid analogues suggests that valyl-$tRNA^{Val}$ is a key component in the derepression mechanism. In E. coli and Salmonella typhimurium, it has been shown that restriction of the supply of a particular amino acid specifically increases the rate of synthesis of its cognate ligase (Nass & Neidhardt, 1967; Williams & Neidhardt, 1969; McGinnis & Williams, 1971; Archibold & Williams, 1972; Gahr & Nass, 1972) and the involvement of $tRNA^{His}$ has been implicated in the control of Salmonella histidine: tRNA ligase (McGinnis & Williams, 1972a b). Therefore amino acids or some derivative seem to be the most likely candidates involved in ligase regulation in plants.

The objects of this thesis were to investigate the role of amino acids in the control of arginine: and leucine: tRNA ligases and to attempt to reach an understanding of the mechanisms underlying the changes in activity of these enzymes which occur during the growth of tobacco XD cells in suspension culture (Wray et al., 1974).

MATERIALS AND METHODS

Chemicals

99 mol% deuterium oxide and 99 mol% ^{15}N-sodium nitrate or 99 mol% ^{15}N-nitric acid (50% w/v) were obtained from Bio-Rad Laboratories Ltd., Bromley, Kent.

^{32}P-orthophosphate (1 mCi/ml), ^{3}H-n-hexadecane (2.27 mCi/g), L-5 ^{3}H-arginine-HCl, (16 Ci/mmol), L-2,5 ^{3}H-histidine (58 Ci/mmol), L-4,5-^{3}H-isoleucine (11 Ci/mmol) and L-4,5-^{3}H-leucine (53 Ci/mmol), were obtained from the Radiochemical Centre, Amersham, Bucks.

2,5 diphenyloxazole (PPO), 1,4-di-2-(5-phenyloxazolyl)-benzene (POPOP) and toluene were obtained from Fisons Ltd., Loughborough, England.

Phase combining system (PCS) was obtained from Amersham/Searle Corpn. Illinois.

Sephadex G25 (coarse) was obtained from Pharmacia, Sweden.

DE 52 Diethylaminoethyl cellulose and glass fibre filter discs GF/A, GF/B, GF/C were obtained from Whatmans Biochemicals, Maidstone, Kent.

Millipore pre-filter glass fibre discs were obtained from Millipore (U.K.) Ltd., Abbey Road, London.

L-amino acids, amino acid analogues and protamine sulphate were obtained from the Sigma London Chemical Company Ltd.

Yeast tRNA was obtained from the Boehringer Corpn. (London) Ltd., Uxbridge.

All other chemicals were of the best quality available from the usual commercial sources.

Plant Material

Suspension cultures of Nicotiana tabacum cells were used for this work. The cell line was derived from parenchyma pith of Turkish tobacco, Nicotiana tabacum var. Xanthi by Dr. Philip Filner in 1961. Stem sections were removed from plants and a callus was produced when placed on nutrient agar containing M-1D , malt extract and casein

hydrolysate.

The XD line was established by subculturing into a completely defined medium (M-1D) in 1963.

Composition of M-1D Medium

M-1D medium was prepared by taking aliquots of the stock solutions listed in Table 3 and diluting with distilled water to 1 litre. After the addition of sucrose, the medium was adjusted to pH 6.2-6.5 with 1M NaOH.

For enzyme and growth studies the cells were grown in either 250 ml or 500 ml Ehrlenmeyer (conical) flasks containing 100 ml or 200 ml of M-1D medium respectively. For tRNA preparations, 5 litre or 10 litre flasks were used containing 2 litres or 3 litres of M-1D respectively. Flasks were stoppered with foam or cotton wool plugs, capped with aluminium foil and autoclaved at 20 psi for 15 min. Maintenance of the culture by subculturing was routinely performed every 14 days by pipetting an aliquot of stationary phase cells into fresh medium (0.4g/100ml). Flasks were shaken on an orbital shaker so that cells were kept in suspension at 27°.

Addition of amino acids and amino acid analogues was made prior to autoclaving and the pH re-adjusted to 6.2-6.5 if required.

Growth Assays (Fresh Weight Determination)

Cells were harvested by filtration under light suction on Miracloth (Chikopee Mills Inc. New York) and weighed.

Extraction of Enzymes

(1) For Kinetic and Developmental Studies

1-2g of cells were harvested by filtration on Miracloth, weighed and homogenized in a glass-teflon Potter homogenizer at 4° in 0.1M tris-HCl buffer, pH 7.5 containing 25 mM mercaptoacetate (3ml buffer/g cells). The homogenate was then centrifuged at 39,000 rpm (110,000g) for 1h in an MSE Superspeed 65 preparative ultracentrifuge. The supernatant was

Table 3. Composition of M-1D Medium

Stock solution	Compound	To make 500 ml of stock soln. (g)	To make 1 litre of M-1D (ml)
WM-I	$Ca(NO_3)_2.H_2O$	10.0	10
	KNO_3	4.0	
WM-II	NaH_2PO_4	0.825	10
	Na_2SO_4	10.0	
WM III	$MgSO_4.7H_2O$	18.0	10
	KCl	3.25	
WM IV	$FeCl_6H_5O_7.3H_2O$	1.0	
	$MnSO_4.H_2O$	2.525	
	$ZnSO_4.7H_2O$	0.75	1
	H_3BO_3	0.75	
	KI	0.375	
WM-V	Nicotinic acid	0.25	
	Pyridoxine.HCl	0.05	1
	Thiamine.HCl	0.05	
2-4-D	2,4 dichlorophenoxy-acetic acid	0.05	5
	Sucrose	20.0 g	

Nitrateless M-1D

Solution WM-I was replaced by N^- WMI which contained

$CaCl_2$ 4.73g

and KCl 2.95g

Stock solutions were stored at 4^o

collected and adjusted to 75% saturation with a saturated solution of ammonium sulphate pH 7.5. After stirring for 1h at 4° the precipitated protein was collected by centrifugation at 15,000 rpm (25,000g) in an MSE high speed 18 centrifuge and dissolved in 2 ml of 0.1M tris-HCl buffer, pH 7.5 containing 25 mM mercaptoacetate. After passing through a column (40 x 2cm) of Sephadex G25 (coarse) previously equilibrated with buffer, the fractions containing protein were pooled, suitably diluted with extraction buffer and used as the source of enzyme.

(2) For Density Labelling Studies

After homogenisation and centrifugation at 39,000 rpm (110,000g) as described above, the supernatant was passed through a Sephadex G25 column and the pooled protein fractions adjusted to 75% saturation with saturated ammonium sulphate pH 7.5. After centrifugation at 15,000 rpm (25,000g) the protein precipitate was dissolved in 1.5 ml of 0.1M tris-HCl buffer pH 7.5 containing 25 mM mercaptoacetate and a suitable dilution, equivalent to 2 mg of protein, was used for isopycnic equilibrium centrifugation.

(3) For DEAE Cellulose Column Chromatography

A known weight of cells equivalent to approximately 80 mg of cell protein (ranging from 35g for 2 day enzyme, 40g for 7 day enzyme and 70g for 2 day nitrateless enzyme) was filtered, homogenised (3ml buffer/g cells), and centrifuged at 39,000 rpm (110,000g) as described above. 2% protamine sulphate was added to the resulting supernatant (approximately 25µl/mg nucleic acid, that is 2.5 ml for 7 day old cells and 1.5 ml for 2 day old and 2 day nitrateless cells), and after stirring at 4° for 1h the precipitated nucleic acids were removed by centrifugation at 15,000 rpm (25,000g). Following adjustment of the supernatant to 75% saturation with a saturated solution of ammonium sulphate pH 7.5, the precipitated protein was dissolved in 10 ml of 20mM potassium phosphate buffer pH 7.5 containing 10mM mercaptoethanol and

dialysed against the same buffer for 15h at 4°.

Extraction of tRNA

Approximately 300g of cells were harvested by filtration on Miracloth and weighed. Total RNA was extracted from the cells by the phenol method and tRNA purified from this by a procedure based on that of Vanderhoef et al. (1970).

Ice cold 10mM tris-HCl, pH 7.5 containing 10mM $MgCl_2$ and 1mM cysteine was added to the harvested cells (3ml buffer/g cells) and the slurry homogenised with an equal volume of liquefied phenol (70ml buffer/kg phenol) in a glass-teflon Potter homogeniser. The homogenate was shaken for 1h at room temperature and after centrifugation at 2000 rpm (2000g) in an MSE 6L Mistral centrifuge, the aqueous upper layer was collected. This was extracted twice more with phenol by shaking for 30 min. The final aqueous layer was collected, 0.25 vol of 5M NaCl and 2.5 vol of 95% ethanol were added and total RNA allowed to precipitate at -10° for 15h. The precipitated RNA was collected by centrifugation at 15,000 rpm (25,000g) suspended in 30 ml of 3M sodium acetate pH 6.0 and stirred for 15h at 4° to selectively dissolve the tRNA. After centrifugation at 15,000 rpm (25,000g), the supernatant was collected, the residual material washed twice with 3M sodium acetate, pH 6.0, and after centrifugation the supernatants were combined. 1vol of H_2O and 2.6vol of 95% ethanol were then added and the crude tRNA allowed to precipitate at -10° for 15h. RNA was collected by centrifugation and dissolved in 4 ml of 0.1M sodium acetate buffer pH 7.5. Amino acids were stripped from the tRNA by adding an equal volume of 0.4M tris-HCl pH 8.8 and incubating at 37° for 5h. RNA was precipitated with 0.15 vol of sodium acetate buffer pH 5.0, 0.33 vol of 5M NaCl and 3.2 vol of 95% ethanol for 15h and after centrifugation at 15,000 rpm (25,000g) dissolved in 3 ml of 10mM sodium acetate buffer pH 4.5 containing 10mM $MgCl_2$ and 0.3M NaCl (buffer A). Residual

protein, tRNA and carbohydrate were removed from the tRNA by DEAE cellulose chromatography.

The solution of crude tRNA was placed on a column (10 x 1.5cm) packed with DEAE cellulose, previously equilibrated with buffer A. The column was eluted with 60 ml of buffer A followed by 60 ml of 10mM sodium acetate buffer pH 4.5 containing 10mM $MgCl_2$ and 0.75M NaCl. Fractions containing tRNA, determined from optical density readings at 260 nm, were pooled and after precipitation with 95% ethanol the tRNA was dissolved in 1 ml of 0.1M tris-HCl buffer pH 7.5.

The yield of tRNA was determined by assuming that 1 mg of tRNA was equivalent to an extinction value of 24 at 260 nm (Ehrenstein, 1967).

Pyrolysis of $[^{32}P]$-orthophosphate

All work performed with radioactively labelled $[^{32}P]$-phosphorous was carried out as far as possible behind a 2 cm thick perspex screen. $[^{32}P]$-orthophosphate was converted to $[^{32}P]$-pyrophosphate after the method of Lee Peng (1956).

3 mCi of $[^{32}P]$-orthophosphate (3ml) were transferred, using a 5 ml syringe, into a hydrolysis tube and taken down to dryness under a photoflood lamp. 1 ml of 10mM disodium hydrogen phosphate was then added as carrier and the tube contents again taken down to dryness. The tube was stoppered, wrapped in tin foil and placed in an oven at 500° for 4h and 5 ml of 20mM tetra sodium pyrophosphate was added after cooling. 2 ml of the pyrolysed solution was diluted with 18 ml of 20mM pyrophosphate and used as the working solution.

Assay of Amino Acid : tRNA Ligases

(1) By Aminoacylation of tRNA

Enzymes were assayed by measuring the rate of aminoacylation of tRNA by $[^{3}H]$-amino acids. The incubation mixture contained 30 µmol tris-HCl buffer, pH 7.5, 1 µmol glutathione, 2 µmol mercaptoacetate,

200 µg bovine serum albumen, 2 m µmol [^{3}H]-amino acid (0.2 mCi/µmol), 60-120 µg tRNA, enzyme and appropriate concentrations of ATP and Mg^{2+} in a total volume of 0.2 ml. Incubations were performed at 28°. The rate of aminoacylation for kinetic and developmental studies was measured by removing 20 µl aliquots of incubate at time intervals into ice cold 10% trichloroacetic acid. In the case of density labelling and DEAE cellulose chromatography studies, the rate of aminoacylation was measured by stopping the total reaction mixture with ice cold 10% trichloroacetic acid after a fixed time interval. The resultant precipitate was collected on Millipore or Whatman GF/C filters and washed 6 times with 3 ml of 10% trichloroacetic acid. Filters were then dried at 100° and placed in scintillation vials to which 5 ml of scintillation fluid (0.3g POPOP and 5g PPO/l toluene) was added. Radioactivity on the filters was measured in an Intertechnique Model SL 30 Liquid Scintillation Spectrometer.

Counting Efficiency

This was determined by counting a known activity of [^{3}H]-n-hexadecane in 5 ml of scintillation fluid (0.3g POPOP and 5g PPO/l toluene). For example when 0.0674 µCi of [^{3}H]-n-hexadecane was counted in 5 ml of scintillation fluid 69,984 cpm were recorded. However, this amount of hexadecane is equivalent to 149,890 dpm and so the counting efficiency of ^{3}H under these conditions is

$$\frac{69,984}{149,890} \times 100 \quad \text{i.e. } 47\%$$

The effect of glass fibre filters was determined from a comparison of the cpm obtained in 5 ml of PCS when a known activity of [^{3}H]-amino acid was either dispersed in the fluid or dried onto a GF/C filter. Dispersion of 0.04 µCi of [^{3}H]-arginine in 5 ml of PCS gave 28,470 cpm but when a similar amount was dried onto a GF/C filter and placed into 5 ml of PCS only 25,493 cpm were obtained. The loss in efficiency of counting due to the GF/C filter was therefore

$$\frac{(28,470 - 25,493)}{28,470} \times 100 \quad \text{i.e. } 10\%$$

From the above observations the efficiency with which ^{3}H is counted when placed onto a GF/C filter in 5 ml of scintillation fluid is 42.3%.

The specific activity of $[^{3}H]$-amino acids was accurately determined by comparison with a known activity of $[^{3}H]$-n-hexadecane. For example 0.04 μCi of $[^{3}H]$-n-hexadecane gave 33,043 cpm in 5 ml of PCS, however 0.04 μCi of 50 μM $[^{3}H]$-arginine gave only 28,420 cpm. Therefore the true specific activity of this sample of $[^{3}H]$-arginine was not 0.2 μCi / μmol but $\frac{28,420}{33,043}$ x 0.2 μCi / μmol i.e. 0.17 μCi / μmol.

All the above factors were taken into account when calculating enzyme activity.

(2) By ATP-Pyrophosphate Exchange

This assay was based on appropriate modifications of methods described by Peterson and Fowden, (1965) and Anderson and Fowden, (1969). The reaction mixture contained 2 μmol ATP, 2 μmol $[^{32}P]$-pyrophosphate, 2 μmol amino acid pH 7.5, 50 μmol tris-HCl pH 7.5, 12.5 μmol mercapto-acetate, 10 μmol $MgCl_2$ and enzyme in a total volume of 1.5 ml. The reaction was started by the addition of ATP. After incubation for 10 min. at 28°, the reaction was stopped by the addition of 2 ml of 7.5% trichloroacetic acid. The assay tubes were mixed on a whirlmixer, and 0.5 ml of 10% charcoal in 0.1M pyrophosphate, pH 8.0 was added to each tube (Crane and Lipmann, 1953). After standing for 15h, the charcoal was collected on Whatman No. 1 filter papers and washed 6 times with 4 ml of 0.05M sodium acetate, pH 4.0. The filter papers were then transfered to metal planchettes and radioactivity was determined under a thin end-window Geiger Müller tube (Mullard MX123) operating at 700 volts.

Protein Assay

Protein was assayed by the method of Lowry et al. (1951).

Cells were homogenised in a glass teflon Potter homogeniser at 4° in 0.1M tris-HCl buffer, pH 7.5 containing 25 mM mercaptoacetate (3 ml buffer/g cells) and the homogenate was centrifuged at 39,000 rpm (110,000g) for 1h in an MSE Superspeed 65 preparative ultracentrifuge. A suitable aliquot of supernatant (usually 0.2 ml) was precipitated with an equal volume of 10% trichloroacetic acid in a conical bench centrifuge tube. After 15h at 4° the protein precipitate was centrifuged at 1000 rpm (500g) and the supernatant discarded. The precipitate was washed 3 times with 96% ethanol, dried and dissolved in 1 ml of 1M NaOH.

To 0.2 ml of the test solution, 1 ml of solution C was added with mixing. After 20 min at room temperature, 0.1 ml of Folin-Ciocalteau reagent was added with immediate mixing. After standing for 1h the extinction at 500 nm was read. Protein was determined by comparison with a standard curve containing 0-100 µg of bovine serum albumen.

Reagents A 2% Na_2CO_3 in 0.1M NaOH

B 0.5% $CuSO_4$ $5H_2O$ in 1% sodium tartrate

C 50 parts of A mixed with 1 part of B immediately before use.

Paper Chromatography

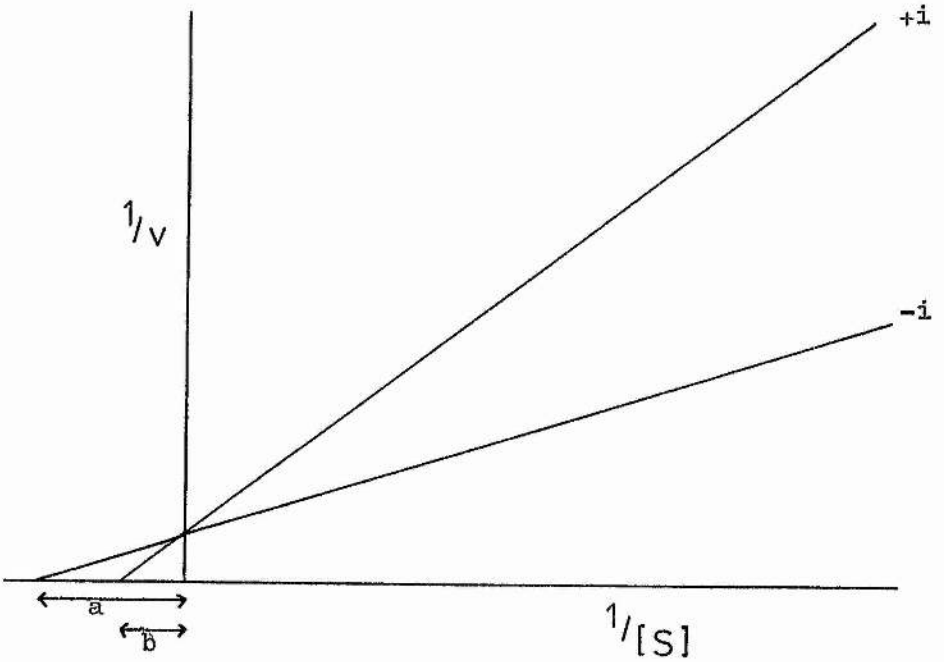
Samples equivalent to 50 µg of amino acid or leucine hydroxamate were spotted onto Whatman No. 1 paper and run in a butanol: acetic acid:water (90:10:29) solvent for 12h. After drying, the chromatograms were sprayed with either 0.5% ninhydrin in butanol, followed by heating at 100° for 10 min (to detect leucine) or $FeCl_3$ $6H_2O$ (5g) in 100 ml of 95% alcoholic 0.1M HCl (to detect hydroxamate, Fishbein et al., 1969).

Km and Ki Studies

For Km determinations, the initial rates of aminoacylation of tRNA by enzyme were obtained at various amino acid concentrations

and Lineweaver-Burk plots plotted.

For Ki determinations, the initial rates were similarly obtained, but in the presence of inhibitor. The value of Ki was derived from Lineweaver-Burk plots as indicated below.



$$a = -\frac{1}{Km} \qquad b = -\frac{1}{Km} / (1 + \frac{i}{Ki})$$

where i = inhibitor concentration

Preparation of Cells for Density Labelling Studies

Cells were fully labelled with heavy isotope by continuous growth for at least 100 generations in M-1D medium containing ^{15}N-nitrate/30% deuterium oxide.

Isopycnic Equilibrium Centrifugation

Isopycnic equilibrium centrifugation was performed in an MSE Superspeed 65 preparative ultracentrifuge using a 3 x 6.5ml titanium swing-out rotor. The gradient mixture had the following composition:-

1ml saturated solution of CsCl prepared at 20°

1 ml 0.1M tris-HCl buffer, pH 7.5, containing 25mM mercaptoacetate

1ml enzyme (approximately 2mg of protein)

The mixture was layered over with 1.5 ml of liquid paraffin.

Following centrifugation at 39,000 rpm (110,000g) for 65h at 4° the tubes were punctured through the bottom and about 75 fractions of 3 drops each (0.04 ml) were collected. Every 5th fraction was used for refractive index determinations using a Bellingham and Stanley Abbé type refractometer. Buoyant density was determined from the following equation:-

$Q^{25} = (10.8601 \times \text{Refractive Index}) - 13.4974$ (Anon, 1970)

where Q^{25} = density at 25°

Ligase activity was determined in the other tubes either by ATP-pyrophosphate exchange or by aminoacylation of tRNA. In the former, the reaction was started by the addition of 0.1 ml of ATP and stopped with 2 ml of 7.5% trichloroacetic acid after a 10 min incubation. In the latter, incubation mixture was added to a final volume of 0.2 ml and the total reaction mixture stopped with 3 ml of 10% trichloroacetic acid after a suitable time interval. This time interval was chosen so that tRNA was not saturated with amino acid during the incubation period, thereby ensuring that initial rates of aminoacylation were measured.

In order to determine this time interval therefore, a fraction thought to be in the peak area and thus possessing a high enzyme activity was incubated for 5 min with reaction mixture. Based on the level of activity obtained and the known saturation level of 120 µg of tRNA, a suitable incubation time could be chosen for the assay of the remaining tubes.

Peak centres and half peak widths were determined from Gaussian plots of the equilibrium centrifugation profiles using Gaussian graph paper (Onno, 1961).

Calibration of Mixing Vessels for DEAE Cellulose Chromatography

The mixing chamber contained 120 ml of distilled water and the reservoir 120 ml of 30 μM methylene blue. 3 ml fractions were collected and absorbance readings determined at 600 nm.

Preparation of DEAE Cellulose for Chromatography

DEAE cellulose was stirred into 15 vol by weight of 0.5M HCl and left to stand for 45 min. The supernatant liquor was then decanted and the slurry adjusted to pH 4.0 by dropwise addition of 1M NaOH. The DEAE cellulose was then stirred into 15 vol of 0.5M NaOH and left for a further 45 min. The supernatant was decanted and the DEAE cellulose stirred into the required buffer (15 ml/g dry wt.). The buffer pH was readjusted by addition of acid if necessary.

Fractionation of Arginine: and Leucine : tRNA Ligases on DEAE Cellulose

DEAE cellulose prepared as above was packed into a column (12 x 1.5 cm) and equilibrated with 20mM potassium phosphate buffer pH 7.5 containing 10mM mercaptoethanol (Buffer 1). The enzyme preparation was then loaded onto DEAE cellulose and washed with 50-60 ml of buffer 1, followed by elution with a linear KCl gradient. The mixing chamber contained 120 ml of buffer 1 and the reservoir appropriate concentrations of KCl in 120 ml of buffer 1. Approximately 80 3 ml fractions were collected and a suitable aliquot assayed for ligase activity by addition of incubation mixture to a final volume of 0.2 ml. The incubation time was chosen in a similar manner to that described for the density labelling studies. Elution of protein was followed by monitoring absorbance readings of the fractions at 280nm.

RESULTS AND DISCUSSION

SECTION I

DEVELOPMENT OF ASSAY FOR ESTIMATION OF ARGININE: AND LEUCINE:tRNA LIGASES

INTRODUCTION

Amino acid:tRNA ligases may be assayed by hydroxamate formation (Hoagland, 1955), ATP-pyrophosphate exchange (Berg,1956) or by aminoacylation of tRNA by amino acid (Moldave, 1963).

1. Hydroxamate formation.

$$\text{Enzyme} + \text{ATP} + \text{Amino acid} \rightleftharpoons (\text{Aminoacyl-AMP-Enzyme}) + \text{PPi}$$

$$\Big\downarrow\ NH_2OH$$

$$(\text{Aminoacylhydroxamate-Enzyme}) + \text{AMP}$$

The reaction of hydroxylamine, amino acid and ATP with enzyme results in the formation of an aminoacylhydroxamate which can be followed using a ferric chloride colour reaction.

2. ATP-Pyrophosphate exchange.

$$\text{Enzyme} + \text{ATP} + \text{Amino acid} \rightleftharpoons (\text{Aminoacyl-AMP-Enzyme}) + \text{PPi}$$

$$\Big\downarrow\ {}^{32}\text{PPi}$$

$$\text{Enzyme} + \text{Amino acid} + \text{[}{}^{32}\underline{\text{P}}\text{]ATP}$$

The presence of the appropriate amino acid and enzyme results in the formation of an enzyme bound aminoacyladenylate. The equilibrium of the reaction lies in the direction of ATP re-synthesis and therefore addition of ^{32}PPi will result in an exchange of ^{32}P with the terminal 2 phosphorous atoms of ATP. Labelled ATP can be collected on charcoal and radioactivity determined.

3. Aminoacylation of tRNA by amino acid.

$$\text{Enzyme} + \text{Amino acid} + \text{ATP} \rightleftharpoons (\text{Aminoacyl-AMP-Enzyme}) + \text{PPi}$$

$$\Big\downarrow\ \text{tRNA}$$

$$\text{Aminoacyl-tRNA} + \text{AMP} + \text{Enzyme}$$

This assay proceedure measures the physiological reaction and the use of labelled amino acid allows the formation of aminoacyl tRNA to be followed. Both the ATP-pyrophosphate exchange and hydroxamate assays measure only the activation step but as already discussed tRNA

may be a pre-requisite for activation, suggesting either that an enzyme-ATP-amino acid-tRNA complex is essential, or tRNA itself acts as an enzyme activator. In addition, there is a lack of strict specificity for amino acid in the activation step. The ATP-pyrophosphate exchange assay often exhibits a high endogenous activity due to the presence of amino acids in impure preparations or to the liberation of amino acids by proteolysis. ATP-ases may also be a problem. Reproducibility is often poor and there is a need for shielding against the high energy β emission of ^{32}P, a factor which is not advantageous to the processing of a large number of samples.

In general the assay involving aminoacylation of tRNA by amino acid has none of these disadvantages and above all it measures the physiological reaction. However the rate and extent of aminoacylation are extremely sensitive to salt concentration and can be reduced to less than 5% of maximum activity (Loftfield, 1972). Buffers and organic solvents may interfere with enzyme activity (Eigner & Loftfield, 1974) and in addition optimum conditions for the detection or assay of one ligase may be very different from those for another ligase in the same tissue.

Although the aminoacylation assay can be used when enzyme and tRNA are derived from different sources, as the evolutionary gap between the source species widens, the degree of recognition between tRNA and ligase usually decreases. For example the rate of aminoacylation of pea root tRNA by ligase from pea root and other plant sources is similar, whereas tRNA from fungi and bacteria supports a much slower aminoacylation rate with these plant enzymes (Vanderhoef et al., 1972). In some cases erroneous aminoacylation may occur, for example, aminoacylation of $tRNA^{Val}$ from *E. coli* by phenylalanine:tRNA ligase from *N. crassa* (Jacobson, 1971). Recognition of tRNA by amino acid: tRNA ligases is therefore most likely to occur under homologous assay conditions, that is where both tRNA and ligase are derived from

the same source.

The aminoacylation assay was chosen for this thesis because it was felt that the assay measuring the physiological reaction was preferable, especially as one of the enzymes we were going to study, arginine: tRNA ligase, requires the presence of its cognate tRNA for activity and furthermore initial experiments employing the ATP - pyrophosphate exchange assay revealed poor reproducibility.

In order to employ the aminoacylation assay it was necessary to extract tRNA from our tobacco XD cells. Any method employed for this purpose has to remove proteins, carbohydrate and other nucleic acids and yet minimise damage to tRNA from physical shock and degradative enzymes. The most widely used method to de-proteinise tissue is by extraction with phenol and was first used to extract tRNA from rat liver (Kirby, 1956). However this method has several disadvantages, for example there may not be complete removal of protein (Loening, 1967, Vanderhoef et al., 1970) and ribonuclease may still be active (Ralph & Bellamy, 1964), although the latter can be overcome by the addition of bentonite (Brownhill et al., 1959, Cherry & Chrobozek, 1965). Multiple phenol extractions are often required sometimes with the formation of emulsions and RNA can be lost at the phenol/buffer interface (Robbins & Raacke, 1968).

Other methods of de-proteinisation involve the use of detergents such as SDS (sodium lauryl sulphate) either on their own (Rammler et al., 1965, Robbins & Raacke, 1968) or in combination with phenol (Ingle et al., 1965). A method that has received a lot of attention is the use of DEP (diethyl pyrocarbonate) which is able to inhibit ribonuclease (Fedorscák & Ehrenberg, 1966) by reacting with tryptophan (Rosen & Fedorcsák, 1966) and histidine (Melchior & Fahrney, 1970) residues in protein but without affecting RNA or DNA (Fedorcsák & Ehrenberg, 1966). RNA from bean and tobacco leaves extracted with DEP showed similar sedimentation profiles and levels of protein contamination to tRNA extracted by the phenol method

(Solymosy et al., 1968). However although increased yields of total RNA are observed, tRNA yields were lower (Lázár et al., 1969). DEP also promotes the ring opening of adenine (Leonard et al., 1970) and loss of amino acid acceptor ability (Ortwerth, 1971).

Once total RNA has been extracted from the tissue there are a number of methods available to separate low molecular weight species, i.e. tRNA and 5S rRNA from other high molecular weight species. For example (1) Methylated Albumin Kieselguhr column chromatography (Mandell & Hershey, 1966), (2) sucrose gradient centrifugation (Britten & Roberts, 1960), (3) Gel filtration (Delihas & Staehelin, 1966), (4) Gel electrophoresis (Loening, 1967), but the approach most used on a preparative scale is that of differential solubility in salt solutions (Zubay, 1962; Kirby, 1965; Vanderhoef et al., 1970). This technique makes use of the fact that low molecular weight RNA is soluble in high concentrations of sodium acetate whereas high molecular weight species are not. Vanderhoef et al. (1970) reported that tRNA prepared in this way is relatively free of other RNA species but does contain some protein and large amounts of carbohydrate. Carbohydrate can be removed either by 2-methoxyethanol (Kirby, 1964) or DEAE cellulose chromatography (Dudock et al., 1969). DEAE cellulose chromatography also seems to remove materials from low molecular weight RNA preparations which inhibit aminoacylation of tRNA (Vanderhoef et al., 1970). As a final step tRNA should be deacylated so that it possesses maximum amino acid accepting ability (Zamecnik et al., 1960).

The method used for tRNA extraction in this thesis is essentially similar to that used by Vanderhoef et al. (1970) involving a phenol extraction, isolation of low molecular weight RNA with 3M sodium acetate, deacylation and a final purification by DEAE cellulose chromatography.

RESULTS AND DISCUSSION

Purification of tRNA by DEAE Cellulose Chromatography

tRNA for use in the aminoacylation assay was routinely extracted from 7 day old cells as described in the materials and methods section. Using tRNA prepared in this way, purification was carried out by adsorbing the crude tRNA preparation onto DEAE cellulose and eluting carbohydrate and protein impurities with 10 mM sodium acetate buffer pH 4.5 containing 10mM magnesium chloride and 0.3M sodium chloride. High absorbance readings were recorded from fractions 3-7 (column A, Table 4) suggesting that these fractions contained the carbohydrate and protein impurities. tRNA was then eluted from the DEAE cellulose with the same buffer but containing 0.75M sodium chloride. The high absorbance readings obtained from fractions 6-13 (column B, Table 4) indicated that tRNA had been eluted.

A typical yield was usually 5 mg tRNA/100g of cells which is similar to that extracted from tobacco callus tissue (Cornelis & de Patoul, 1975). Wray & Brice, (unpublished work) have demonstrated that most tRNA can be extracted from tobacco cells around 4-7 days while lower levels are found just before and after subculture. These changes in tRNA are very similar to those which occur in sycamore cells during batch culture (Short et al., 1969). When tobacco tRNA was examined by sucrose density gradient analysis (Wray & Brice, unpublished work) it sedimented as a single species with the same sedimentation coefficient as an authentic sample of yeast tRNA. Analysis by gel electrophoresis (Wray & Brice, unpublished work), sometimes revealed the presence of a relatively small amount of another RNA species with a mobility slightly greater than that of tRNA. This may have been 5S rRNA. Tobacco tRNA purified in this way was used in all subsequent experiments.

Table 4. Purification of tRNA by DEAE cellulose chromatography

Fraction Number	Absorbance at 260 nm A	B
1	0.003	0.019
2	0.005	0.020
3	0.128	0.019
4	0.680	0.015
5	0.320	0.018
6	0.128	0.200
7	0.085	∞
8	0.081	∞
9	0.062	∞
10	0.050	∞
11	0.060	∞
12	0.035	1.000
13	0.022	0.680
14	0.017	0.520
15	0.012	0.350
16	0.013	0.230
17	0.012	0.175
18	0.013	0.159
19	0.011	0.140
20	0.012	0.130

Column A shows absorbance readings of 3 ml fractions following loading of tRNA onto DEAE cellulose and washing with 60 ml of 10 mM sodium acetate buffer pH 4.5 containing 10 mM magnesium chloride and 0.3M sodium chloride.

Column B shows absorbance readings of 3 ml fractions following elution of tRNA from DEAE cellulose with 10mM sodium acetate buffer pH 4.5 containing 10mM magnesium chloride and 0.75M sodium chloride.

Sephadexing of 110,000g Supernatant

Tissue was extracted as described in the materials and methods section and centrifuged at 110,000g (39,000 rpm). However amino acids had to be removed from the extract since their presence would alter the specific activity of the amino acid used in the assay. This was achieved by passing the 110,000g supernatant through a Sephadex G25 column, and Fig. 2 shows the profile so obtained. Fraction numbers 11-17 were frothy in appearance suggesting that these fractions contained protein. This was confirmed by the high absorbance readings at 280 nm. A second peak, obtained between fractions 25-34 was presumed to be due to absorption by amino acids. Fractions 11-17 were therefore pooled and used as enzyme source.

Detection of Ligase Activity

It has previously been shown using the ATP-pyrophosphate exchange assay that histidine:,isoleucine: and leucine: tRNA ligases were considerably active in tobacco XD cells grown in suspension culture (Wray et al., 1974). Preliminary experiments with the aminoacylation assay, as described in the materials and methods section, indicated that no aminoacylation of tobacco tRNA or. commercially prepared yeast tRNA could be detected by either histidine: or isoleucine: tRNA ligases (Table 5). Substantial aminoacylation of tobacco tRNA by leucine: tRNA ligase was observed, although there was very little with yeast tRNA, even when it was checked for de-acylation by incubation for 5h at 37^{o} in tris-HCl buffer, pH 8.8. This emphasises the need for homologous assay conditions where both tRNA and ligase are derived from the same source.

There are several possible reasons why aminoacylation of tRNA by histidine: and isoleucine: tRNA ligases could not be observed. These are (1) low levels of enzyme in the tissue (2)

Fig. 2 Profile of absorbance at 280nm of 110,000g supernatant after Sephadex G 25 gel filtration.

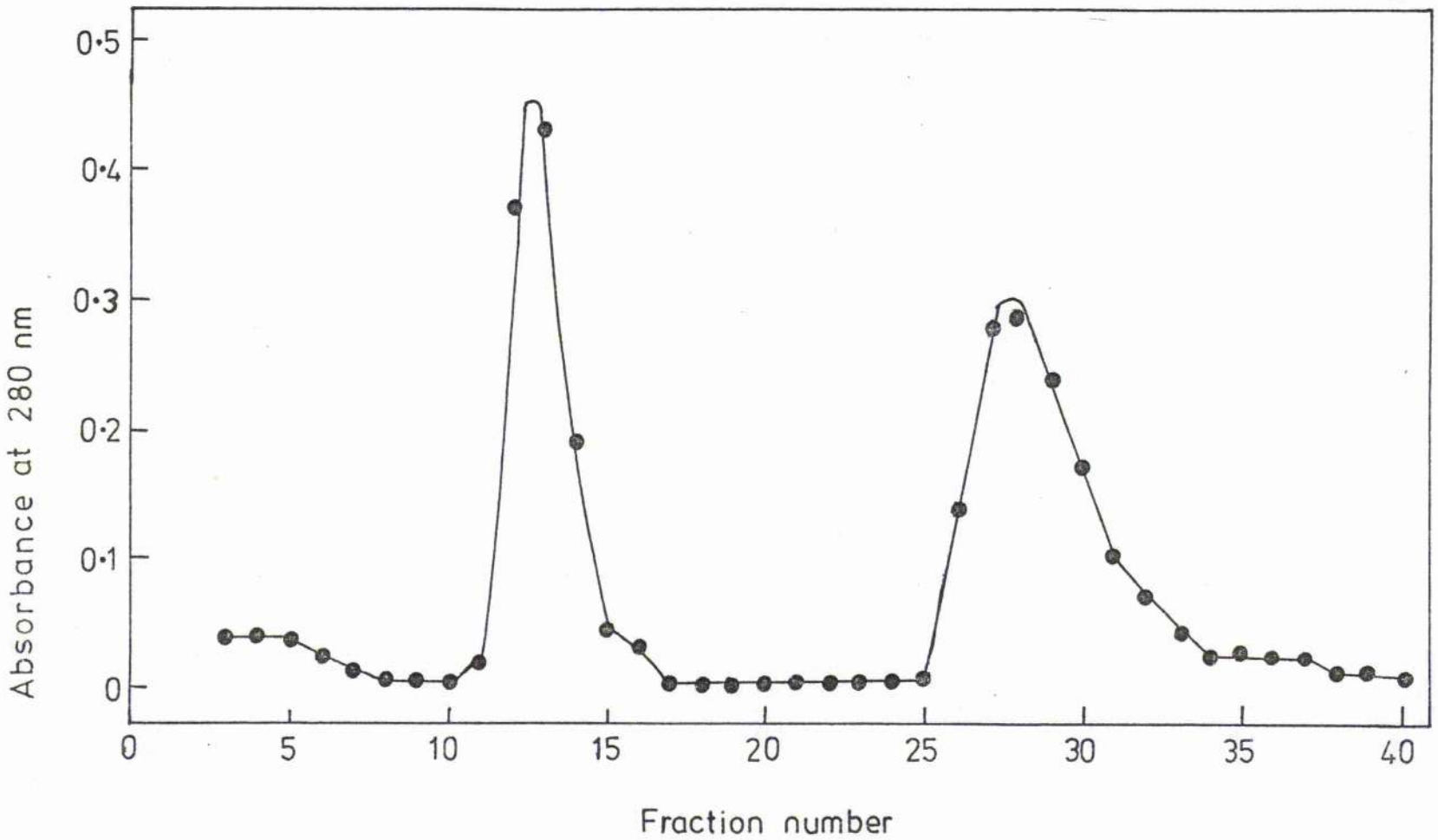

0·5
0·4
0·3
0·2
0·1
0
Absorbance at 280 nm
0
5
10
15
20
25
30
35
40
Fraction number

Table 5. Aminoacylation of tobacco tRNA and yeast tRNA by histidine:, isoleucine:, and leucine: tRNA ligases.

amino acid: tRNA ligase	tRNA (60 µg)	cpm/g cells following a 15min incubation
histidine	yeast	60
histidine	tobacco	54
isoleucine	yeast	37
isoleucine	tobacco	62
leucine	yeast	156
leucine	tobacco	5266
leucine	deacylated yeast	160

Background radioactivity 37 cpm

The reaction mixture as described in the materials and methods section contained 1 µmol Mg^{2+}, 1 µmol ATP and 60 µg tRNA. The total reaction mixture was stopped by addition of 10% trichloroacetic acid after a 15 min incubation period.

inactivation of enzyme during extraction (3) an unfavourable Mg^{2+}/ATP ratio in the reaction mixture (4) Damage to $tRNA^{His}$ and $tRNA^{Isoleu}$ during tRNA extraction.

Subsequently, however, it was established that the Mg^{2+}/ATP ratio was the important factor since, by using Mg^{2+}/ATP ratios of 1,2,4 and 10, aminoacylation of tobacco tRNA could be demonstrated with alanine, arginine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, tryptophan and valine (Wray, Gore & Brice, unpublished work).

Choice of Filters

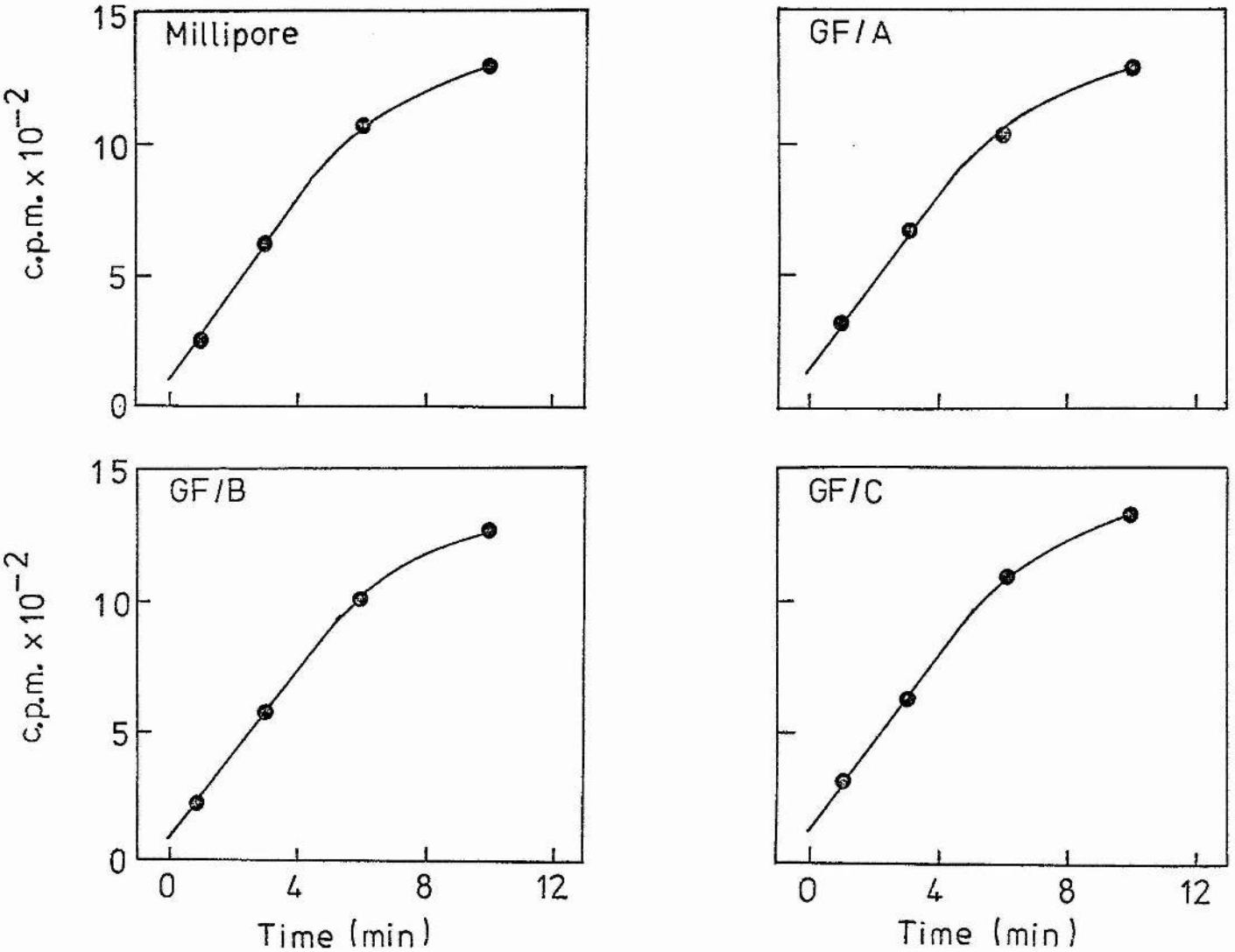
Detection of amino acid: tRNA ligases requires the aminoacylated tRNA to be trapped onto glass fibre filters prior to counting in a liquid scintillation spectrometer. The rate of aminoacylation of tRNA by $[^{3}H]$-leucine was compared using four types of filters whose specifications, as indicated by the manufacturer, are listed below:-

Filter	Diameter (mm)	Thickness (mm)	
Millipore pre-filter	19	0.28	
Whatman GF/A	21	0.20	increasing
GF/B	21	0.65	retention
GF/C	21	0.20	↓ properties

Pore size in all cases was 0.5 μm

Fig. 3 clearly shows that the rate of aminoacylation determined using these different filters was identical. Since the filtration rates differed, that is GF/C > GF/A = Millipore > GF/B, in all subsequent experiments GF/C filters were used as the faster rate of filtration through this type of filter assisted the processing of large numbers of samples.

Fig. 3 Kinetics of aminoacylation of tRNA with [^{3}H]- leucine determined using either Millipore or Whatman GF/A, GF/B and GF/C glass fibre filters.


Millipore
GF/A
GF/B
GF/C
c.p.m. x 10⁻²
15
10
5
0
0
4
8
12
Time (min)

Stability of Leucyl-tRNA^{LEU} in 10% Trichloroacetic Acid

In assays to determine leucine tRNA: ligase activity it was noted that if stopped incubation mixes were left overnight at room temperature then no radioactivity could subsequently be detected on GF/C filters after filtration. It was thought that this might be due to the 10% trichloroacetic acid used to stop the assay reaction and the following experiment was set up to check this. Five tubes containing reaction mixture were incubated for 4 min and the reaction stopped by the addition of 10% trichloroacetic acid. The initial radioactivity was obtained by immediate filtering of one of the tubes. Two tubes were then placed in ice and two left at room temperature and at intervals of 1 and 2h the contents of 1 tube from each pair was filtered and the radioactivity determined.

Fig. 4 shows that at 0^{o} the activity remains relatively constant whereas after 2h at room temperature 75% of the activity is lost.

It would appear that the ester bond is unstable in 10% trichloroacetic acid at room temperature. It is known that this bond is alkali labile being susceptable to tris-HCl buffer, pH 8.8 and hydroxylamine (Berg & Ofengand, 1958). However there appear to be no reports of its stability in trichloroacetic acid, although most investigators using this assay technique indicate that cold trichloroacetic acid should be employed.

Proportionality of the Extent of Aminoacylation to tRNA Level

Fig. 5 demonstrates that the extent of aminoacylation of tRNA by [^{3}H]-leucine was proportional to the level of tRNA used in the reaction mixture. Degradation of tRNA by endogenous ribonuclease did not appear to be a problem since the extent of aminoacylation after establishment of a plateau value was constant for at least 10 min, unlike the situation in aged carrot discs where the plateau value

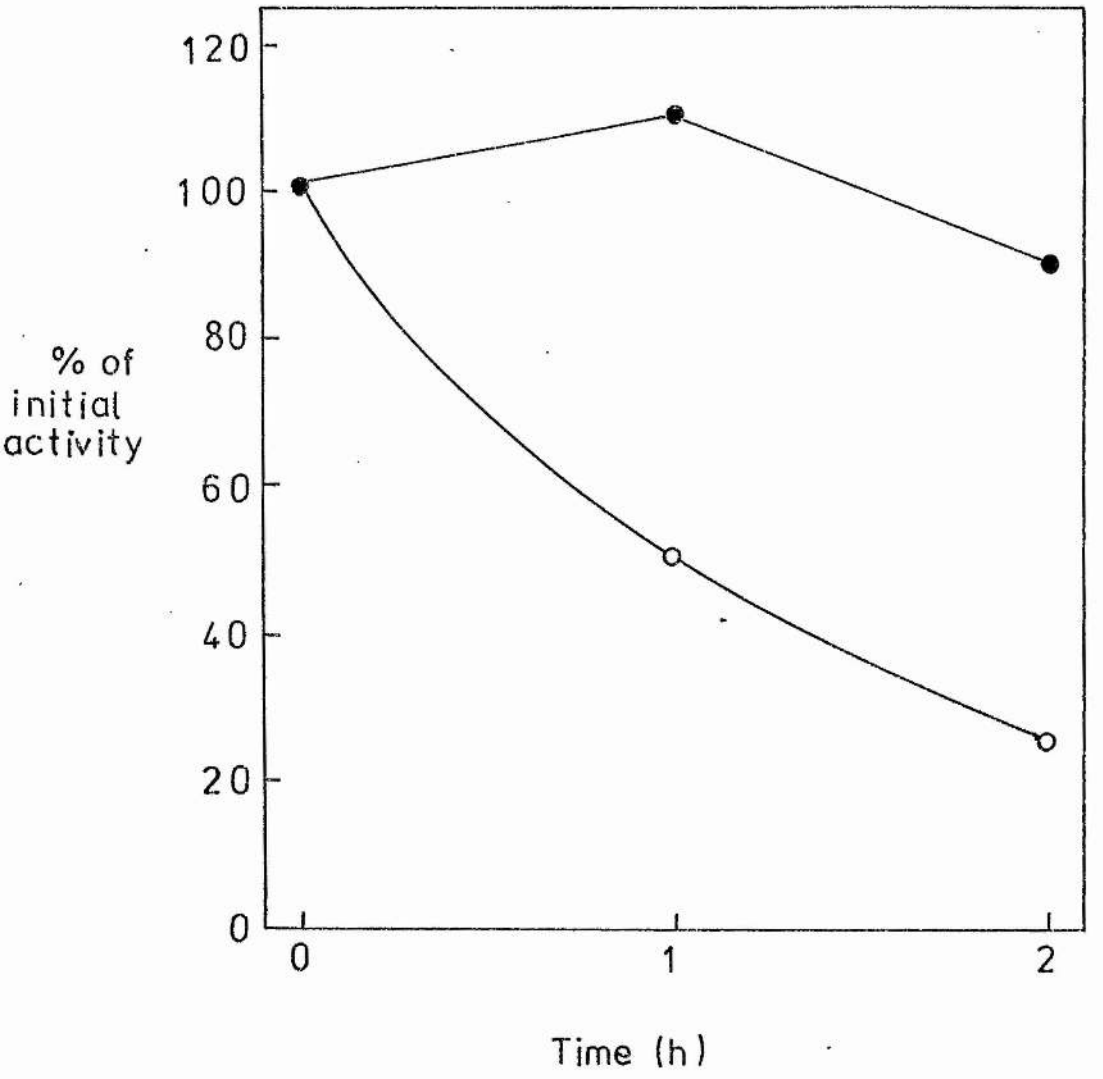


Fig. 4 Stability of leucyl-tRNA^{Leu} in 10% trichloroacetic acid at room temperature ○—○ or at 0° ●—●

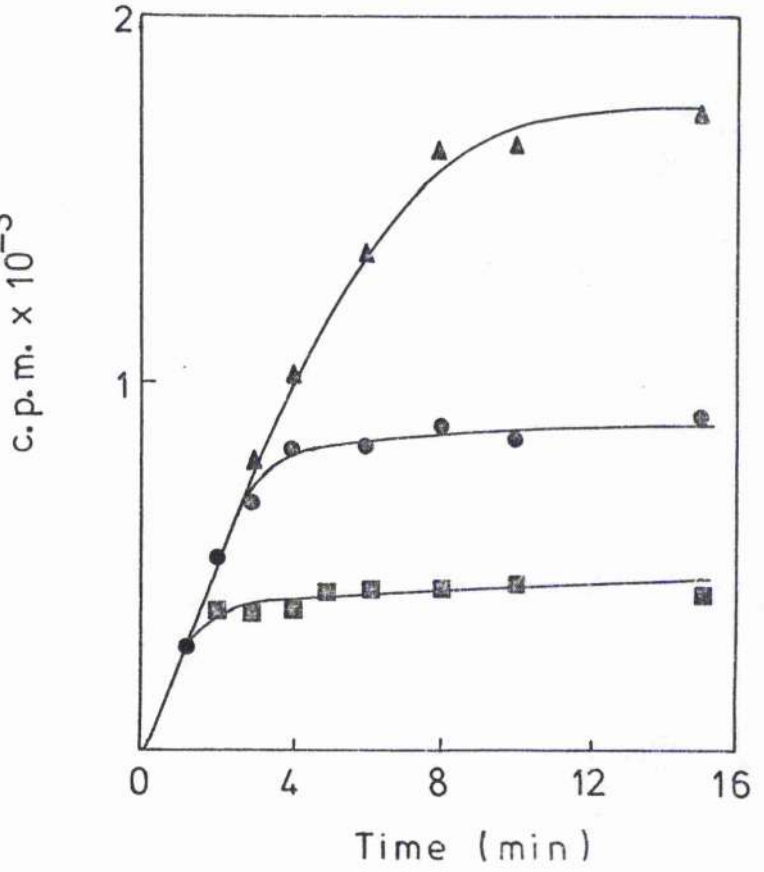


Fig. 5 Kinetics of aminoacylation of tRNA with [^{3}H]- leucine at different tRNA levels.

40µg tRNA ■—■

80µg tRNA ●—●

160µg tRNA ▲—▲

slowly falls (Brierley & Wray, unpublished work). A similar result was obtained with the arginine enzyme and unless otherwise stated experiments described in this thesis used 120 µg of tRNA in the reaction mixture.

Proportionality of Aminoacylation Rate to Enzyme Concentration

The initial rate of aminoacylation of tRNA by leucine: tRNA ligase was measured at different enzyme concentrations (Fig. 6). The rate of aminoacylation increases linearly with enzyme concentration. This has also been demonstrated for arginine: tRNA ligase in these cells and similar reports have come from ligases extracted from carrot discs (Brierley & Wray, unpublished work) and wheat germ (Moustafa & Lyttleton, 1963).

In most developmental studies described in the thesis an enzyme concentration equivalent to 50-60 µg protein was used.

Optimisation of the Magnesium and ATP levels for Arginine: and Leucine: tRNA Ligases

The initial rates of aminoacylation of tRNA were determined at several different ratios of Mg^{2+}/ATP for arginine: and leucine: tRNA ligases by holding the ATP concentration constant and altering the Mg^{2+} concentration. This was done at several different ATP concentrations. The initial rates so obtained were plotted against ATP concentration (Fig. 7).

An examination of the data shows that arginine: and leucine: tRNA ligases have different optimal Mg^{2+}/ATP ratios; for arginine it is 2:1 and for leucine 1:1. Another point is that as the absolute level of Mg^{2+} and ATP within a particular ratio is increased, there is usually an increase in enzyme activity. Thus the optimal activity is dependent not only on the Mg^{2+}/ATP ratio but also on the concentration of Mg^{2+} and ATP within that ratio. Dependence of aminoacylation rates on Mg^{2+}/ATP ratios in higher

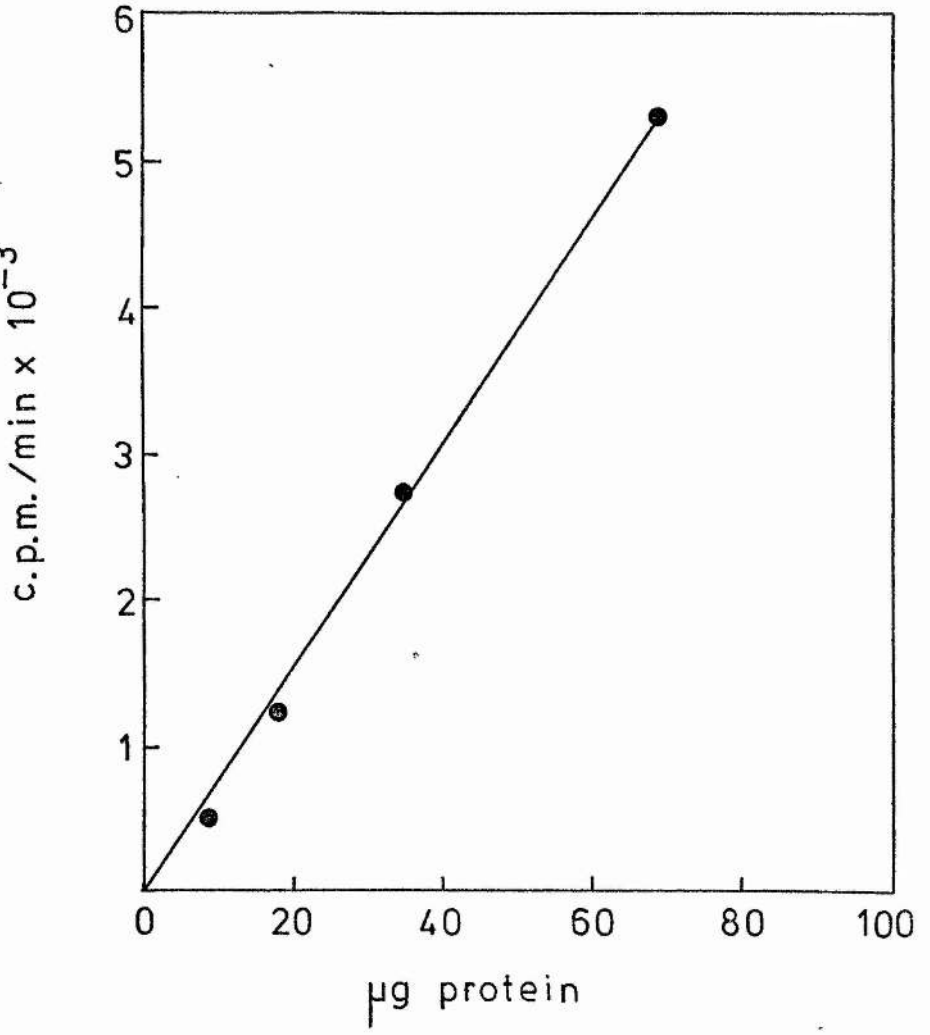
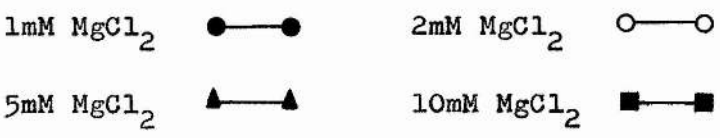
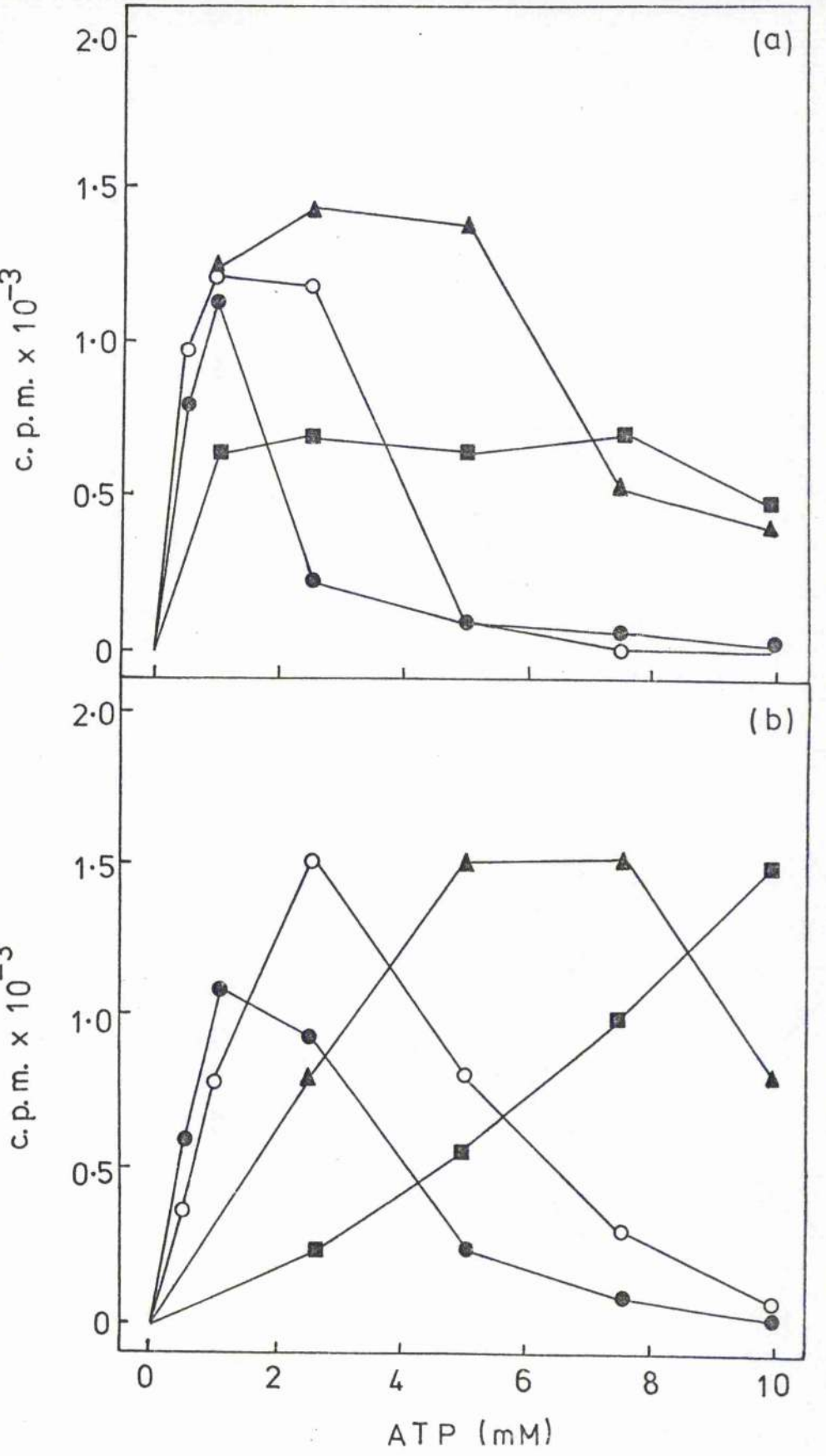


Fig. 6 Initial rate of aminoacylation of tRNA with [^{3}H]- leucine at different enzyme concentrations.

Fig. 7 Dependence of the initial rate of aminoacylation of tRNA on different magnesium and ATP concentrations both for (a) arginine: tRNA ligase and (b) leucine: tRNA ligase.




(a)
(b)
2·0
1·5
1·0
0·5
0
c.p.m. x 10^{-3}
0
2
4
6
8
10
ATP (mM)

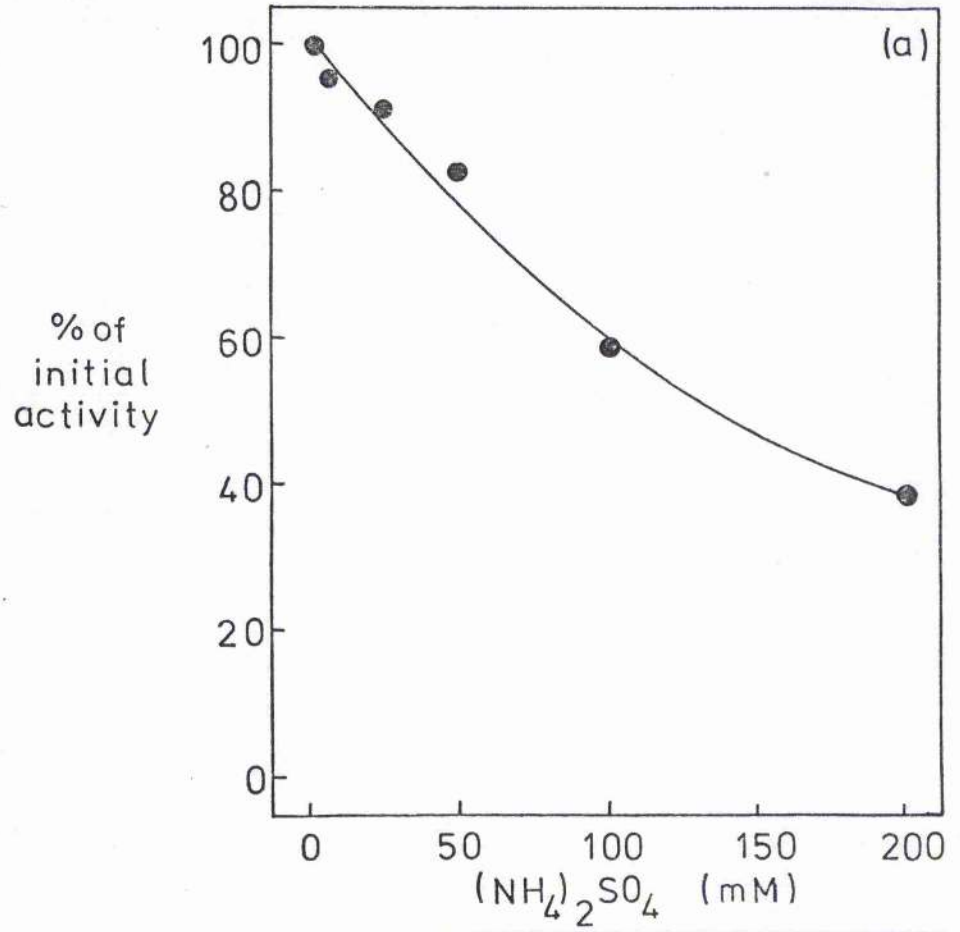
plants has been reported earlier in the studies of Hall & Tao (1970), Burkard et al. (1970) and Cornelis & de Patoul (1975). Similar observations have been made in E. coli and rat liver (Novelli, 1967). It is obviously of greatest importance to determine the optimum Mg^{2+} and ATP concentrations for each individual enzyme and not to use arbitrary concentrations that may allow the optimal detection of some ligase activities but not of others. Thus in all subsequent studies involving arginine: and leucine: tRNA ligases the reaction mixture contained 1 µmol Mg^{2+} and 0.5 µmol of ATP in the case of arginine and 1 µmol Mg^{2+} and 1 µmol of ATP in the case of leucine.

Effects of Ammonium Sulphate and Caesium Chloride on the Activity of Arginine: and Leucine: tRNA ligases

Since ammonium sulphate is involved in the extraction procedure of arginine: and leucine: tRNA ligases, and caesium chloride was to be used in density labelling experiments, it was of interest to look at the effect of these salts on enzyme activity. The initial rates of aminoacylation of tRNA were therefore determined in the presence of different concentrations of ammonium sulphate (Fig. 8) and caesium chloride (Fig. 9). The leucine enzyme was more sensitive to ammonium sulphate than arginine but the reverse was the case for caesium chloride. Caesium chloride inhibition was reversible (Table 6) since in the presence of 1.5M caesium chloride, enzyme activity was totally inhibited compared with control enzyme in normal extraction buffer. However following dialysis of both enzyme samples against 0.1M tris-HCl buffer, pH 7.5 containing 25 mM mercaptoacetate for 15h to remove caesium chloride, 100% of the control activity was regained. Notice also that dialysis of the control enzymes resulted in a 25% loss of enzyme activity.

These results seem to be consistent with other reports. For example leucine: tRNA ligase extracted from Aesculus hippocastanum

Fig. 8 Inhibition of (a) arginine: tRNA ligase and (b) leucine: tRNA ligase by ammonium sulphate.


(a)
100
80
60
40
20
0
% of initial activity
0
50
100
150
200
$(NH_4)_2SO_4$ (mM)

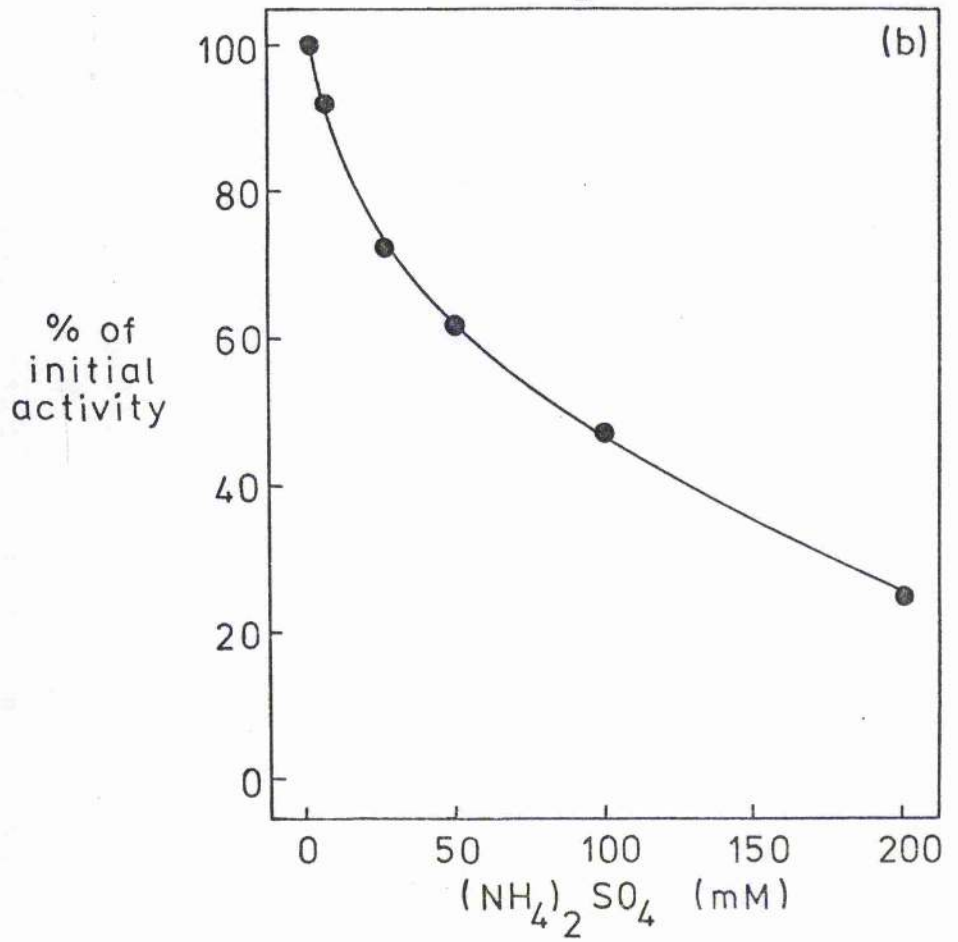
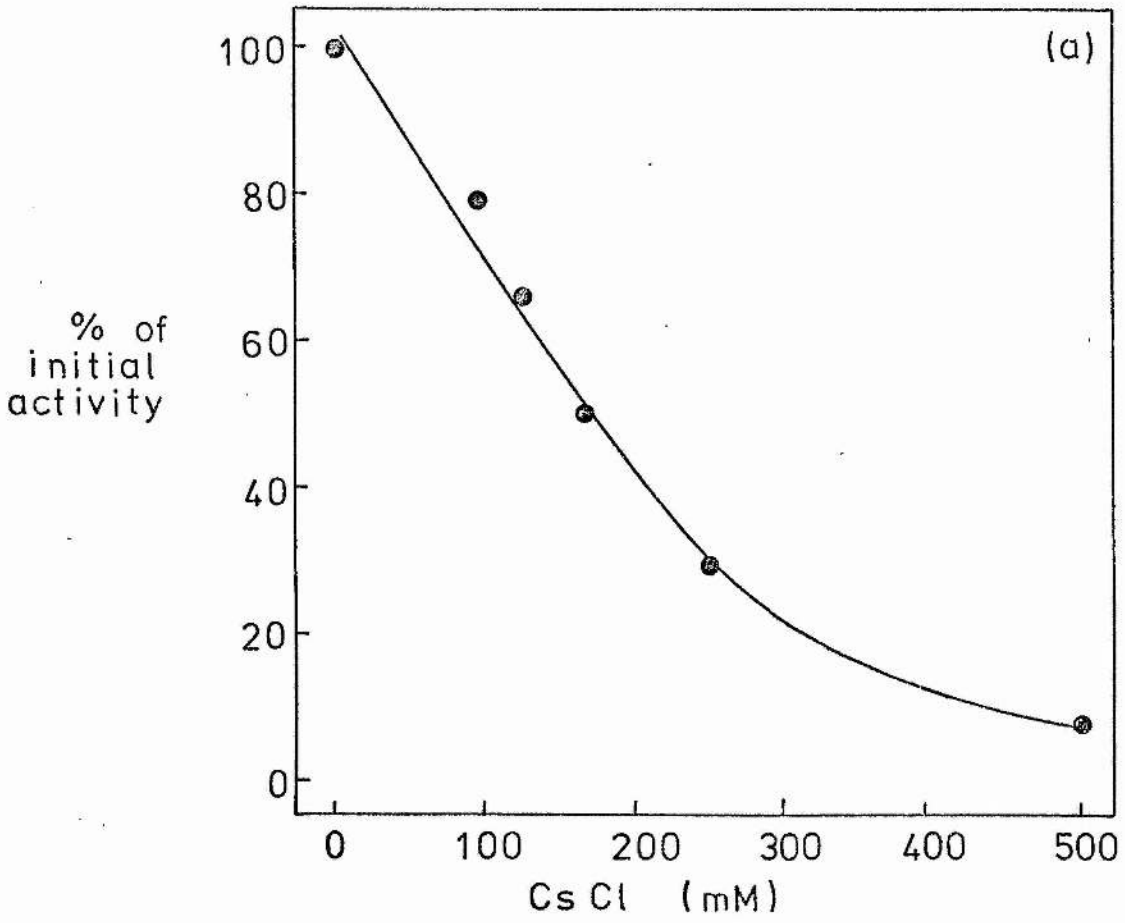

(b)
100
80
60
40
20
0
% of initial activity
0
50
100
150
200
$(NH_4)_2SO_4$ (mM)

Fig. 9 Inhibition of (a) arginine: tRNA ligase and (b) leucine: tRNA ligase by caesium chloride.


(a)
100
80
60
40
20
0
% of initial activity
0
100
200
300
400
500
Cs Cl (mM)

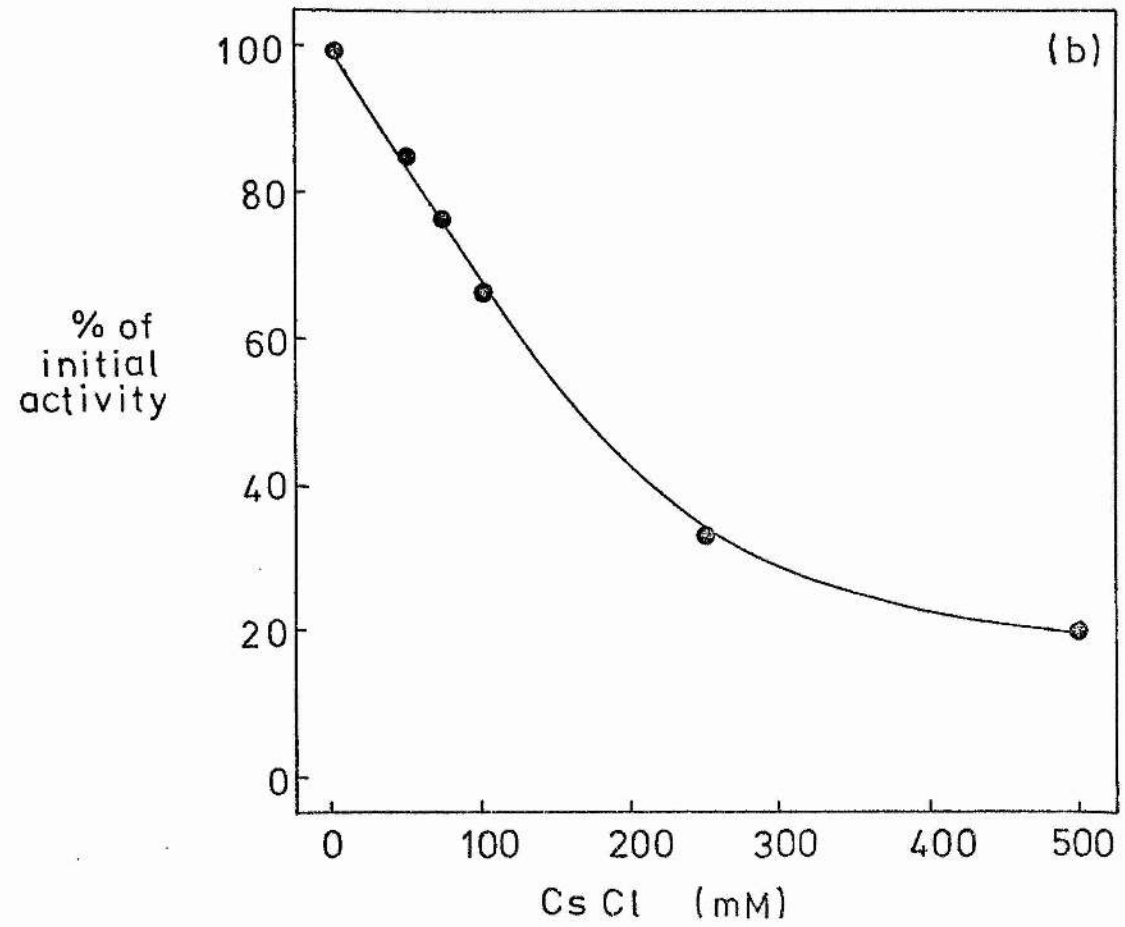

(b)
100
80
60
40
20
0
% of initial activity
0
100
200
300
400
500
Cs Cl (mM)

Table 6. Ligase activity before and after dialysis to demonstrate the reversibility of caesium chloride inhibition

Before dialysis	Activity (nmol/min/g fresh wt)	
	Arginine	Leucine
Control (Enzyme in normal extraction buffer)	0.88	0.37
Test (Enzyme in presence of 1.5M caesium chloride)	-	-
After dialysis		
Control	0.60	0.23
Test	0.63	0.24

(Anderson & Fowden, 1970) is inhibited by a number of salts including caesium chloride and ammonium chloride. Similar effects of salts on ligases have been reported in lupin seeds (Jakubowski & Pawelkiewicz, 1974), E. coli (Peterkofsky et al., 1966; Smith, 1969) and rat liver (Sein & Bećarević, 1971) but the mechanism underlying the inactivation is unclear. It has been suggested that there is an effect on the conformation of ligase (Boyko & Fraser, 1964; Anderson & Fowden, 1970) or of tRNA (Sein & Bećarević, 1971) or of ligase and tRNA (Jakubowski and Pawelkiewicz, 1974). Loftfield (1972), however, reports that tRNA molecules are too rigid for salts to cause conformational changes and proposes that the interaction between tRNA and ligase involves ionic bonds and that the affinity of this interaction is strongly influenced by the di-electric constant of the medium. However, whatever mechanism(s) are involved in salt inhibition, the observation that ATP-pyrophosphate exchange can be inhibited by salts (Boyko & Fraser, 1964; Anderson & Fowden, 1970; Smith, 1969) suggests that inhibition cannot be exerted via an effect on tRNA alone.

Stability of Leucine: tRNA Ligase

Since enzyme was routinely stored at -10°, it was of interest to look at its stability when stored at this temperature. Enzyme was therefore prepared in the normal way and frozen in 1 ml portions at -10°. At various time intervals following preparation, the initial rate of aminoacylation with [^{3}H] -leucine was determined (Fig.10). After 14 days 85% of the initial activity had been lost and significant activity was lost within 24 hours. Consequently in all developmental studies, enzyme activity was determined immediately after preparation. A similar instability was found with arginine: tRNA ligase.

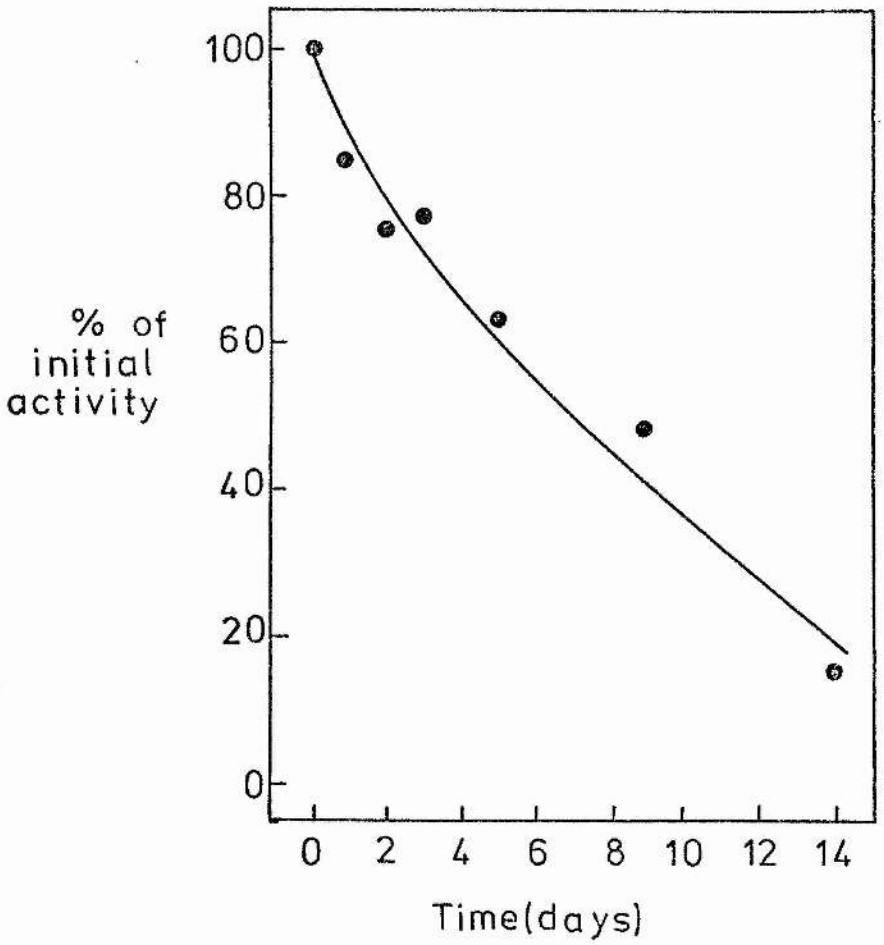


Fig. 10 Stability of leucine: tRNA ligase at -10°.

Determination of the Km Values for Arginine and Leucine

The initial rates of aminoacylation with [^{3}H] -leucine and [^{3}H] -arginine were determined at amino acid concentrations of 1 μM, 2.5 μM, 5.0 μM and 10 μM. Lineweaver-Burk plots of the data gave Km values of 6 μM and 8.3 μM for arginine (Fig.11) and leucine (Fig.12a) respectively.

Following dialysis of the enzyme preparation for 15h against tris-HCl buffer, pH 7.5 containing 25 mM mercaptoacetate at 4°, the Km for leucine was redetermined and found to be unaltered (Fig.12b). This confirms that Sephadex G25 filtration efficiently removes all amino acids from the enzyme preparation, since if they had been present they would have been removed by dialysis. This would have reduced the specific activity of the amino acids used in the assay and thus altered the Km value.

In higher plants there are no reports in which Km values for arginine and leucine have been determined by the aminoacylation assay. In bakers yeast, however a Km value of 2 x 10^{-5}M (20 μM) has been reported for leucine (Chirikjian et al., 1973) and a value of 1.2 x 10^{-6} (1.2 μM) has been reported for arginine in an E. coli mutant (Williams & Williams, 1973). Employing the ATP-pyrophosphate exchange assay a Km for arginine of 7 - 18 x 10^{-5}M (70 - 180 μM) has been reported in Canavalia ensiformis (Fowden & Frankton, 1968), whereas a Km for leucine of 9 μM reported in Aesculus hippocastanum (Anderson & Fowden, 1970) is similar to that found in our studies.

However since amino acid tRNA ligases vary in properties from one tissue to another it is likely that their affinities for amino acids will also vary.

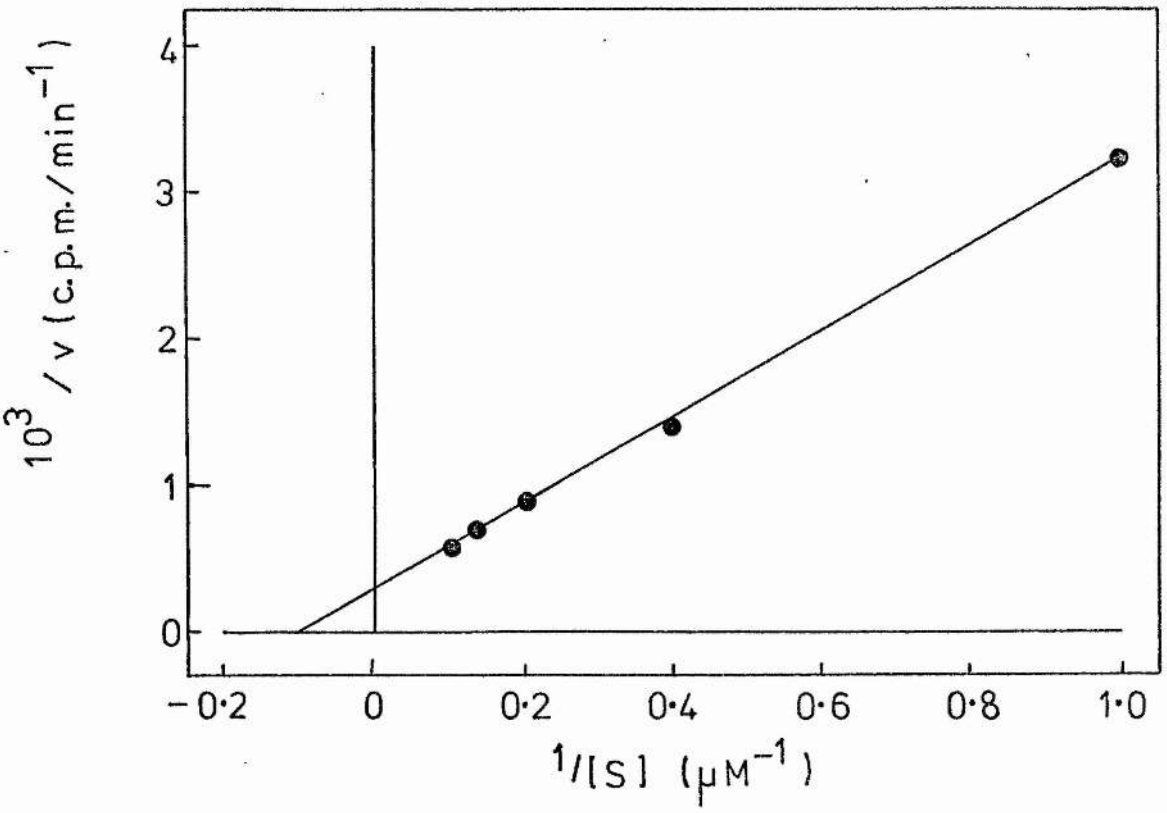
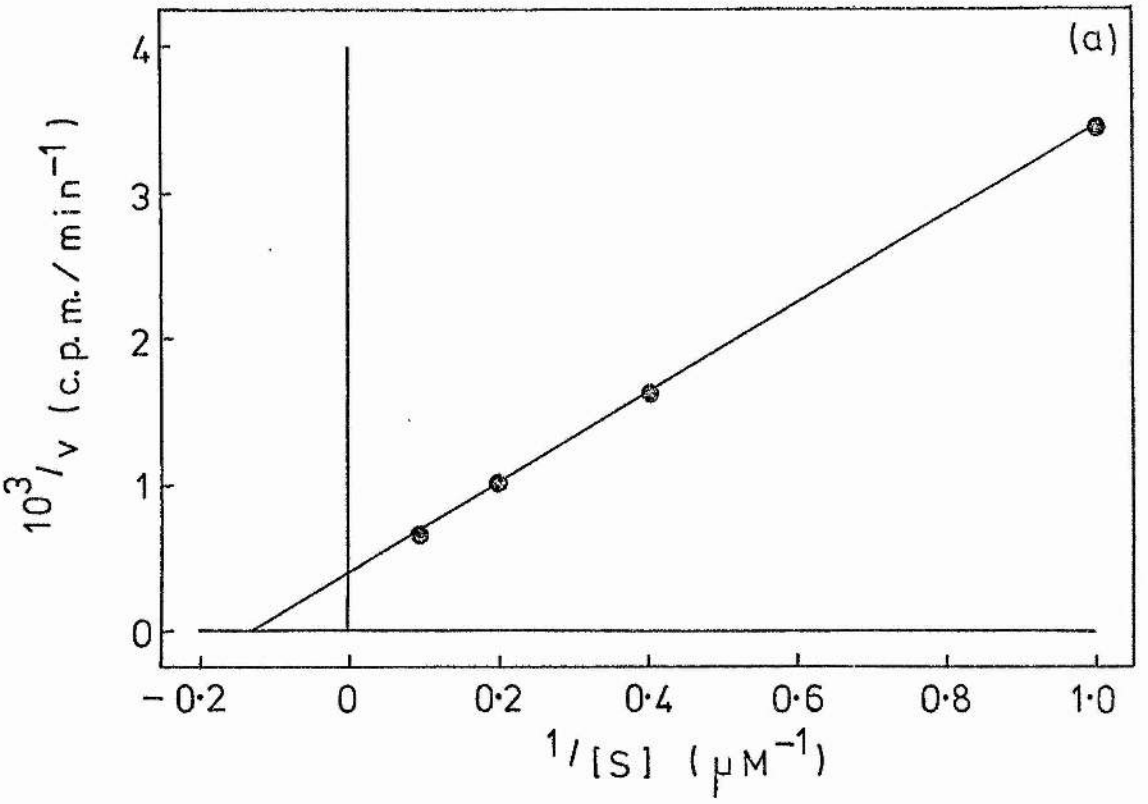
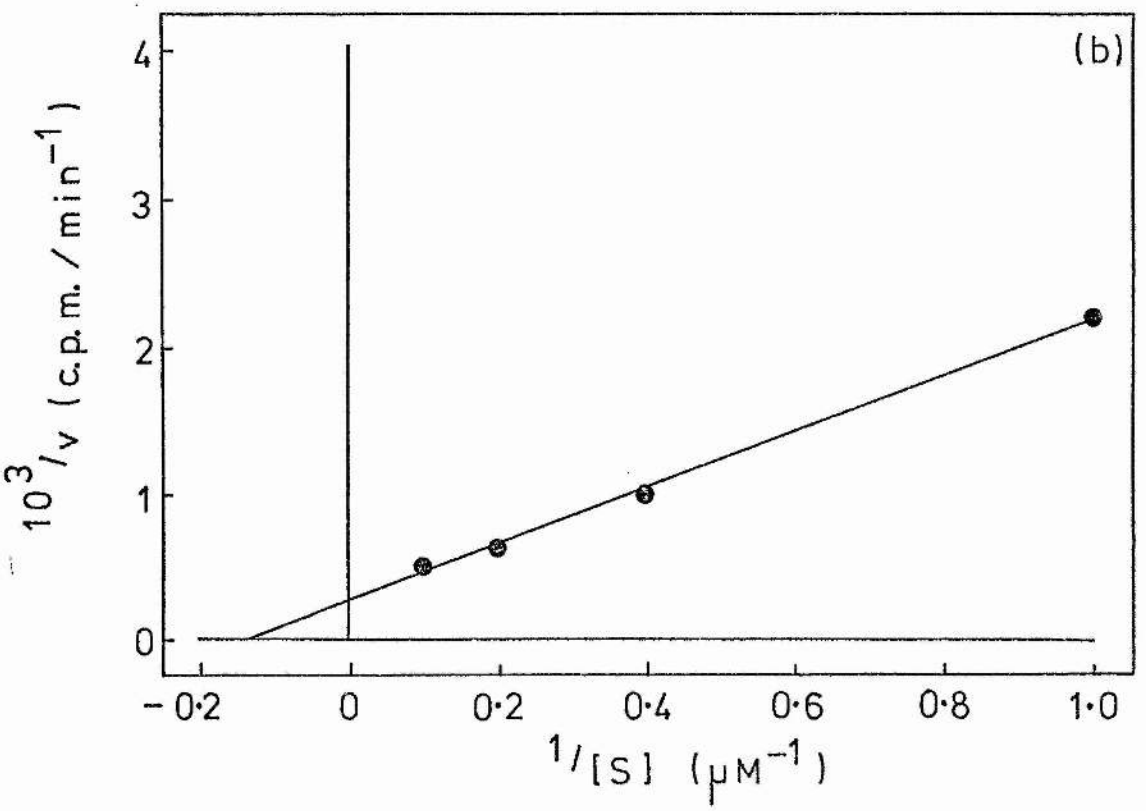


Fig. 11 Lineweaver-Burk plots of the initial rates of aminoacylation of tRNA with [^{3}H]- arginine.

Fig. 12 Lineweaver-Burk plots of the initial rates of aminoacylation of tRNA with [^{3}H]- leucine (a) before dialysis and (b) after dialysis.


(a)
$10^3/v$ (c.p.m./min^{-1})
4
3
2
1
0
−0·2
0
0·2
0·4
0·6
0·8
1·0
1/[S] (μM^{-1})


(b)
$10^3/v$ (c.p.m./min^{-1})
4
3
2
1
0
−0·2
0
0·2
0·4
0·6
0·8
1·0
1/[S] (μM^{-1})

SECTION 2

CONTROL AND DEVELOPMENT OF ARGININE: AND LEUCINE: tRNA LIGASES

INTRODUCTION

In the general introduction we discussed the possible role of amino acids in the regulation of amino acid: tRNA ligases and pointed out that the situation in plants was far from clear. An increase in the free amino acid pool was correlated with an increase in ligase activity by some workers (Henshall & Goodwin, 1964; Wray et al., 1974) but it was not clear whether this increased amino acid pool size led to a general stimulation of protein synthesis or whether there was a specific effect on the synthesis of the ligases. In contrast Anderson & Rowan (1966a)obtained an increase in specific activity of these enzymes along with a decrease in the free amino acid pool.

Two approaches have been adopted in our studies to attempt to show an involvement of amino acids (or some derivative) in the regulation of these enzymes in tobacco XD cells. These approaches are (1) altering the amino acid pool either by subculturing cells into nitrateless medium or by removing arginine from the culture medium of cells grown in the presence of arginine. (2) using amino acid analogues to lower the *in vivo* levels of aminoacyl-tRNA.

We have therefore used the assay technique developed in the previous section to study the changes in arginine: and leucine: tRNA ligases which occur during growth of tobacco XD cells and to determine whether these changes are affected by altering the amino acid pool.

RESULTS AND DISCUSSION

Changes in Fresh Weight and Protein Levels with Culture Age

When stationary phase XD cells are transferred to M-1D, they exhibit a typical sigmoid growth pattern with lag, exponential and stationary phases and protein levels rise to a maximum during early exponential phase (Fig. 13). These results are similar to those determined in a previous study of these cells (Wray et al., 1974), except that the lag phase has shortened and consequently the protein levels peak slightly earlier. Although any number of mutations could account for this observation, it could be that the cells can now utilise nitrate more readily due to a mutation in their nitrate uptake system. This seems very possible as the availability of nitrate is the primary limiting factor for growth. (Filner, 1966).

Variation of Arginine: and Leucine: tRNA Ligases with Culture Age

Using the aminoacylation assay developed in the previous section, the changes in arginine: and leucine: tRNA ligases during growth of tobacco XD cells were followed. Stationary phase cells were transferred to fresh M-1D medium and at several time intervals following subculture, enzyme was extracted and the activity determined from the initial rates of aminoacylation with $[^{3}H]$-amino acid (Fig. 14). The reaction mixture as described in the materials and methods section contained 1 µmol Mg^{2+} and 0.5 µmol ATP for arginine, and 1 µmol Mg^{2+} and 1 µmol ATP for leucine, together with 120 µg of tRNA. There is about a 7 fold increase in both enzyme activities after 4 days although arginine: tRNA ligase is twice as active as the leucine enzyme. These enzymespeak in activity at around 4 days whereas the earlier studies of Wray et al. (1974) show activities peaking at about 6 days. The observation that enzyme peaks 2 days earlier is probably a result of the shortened lag phase discussed above.

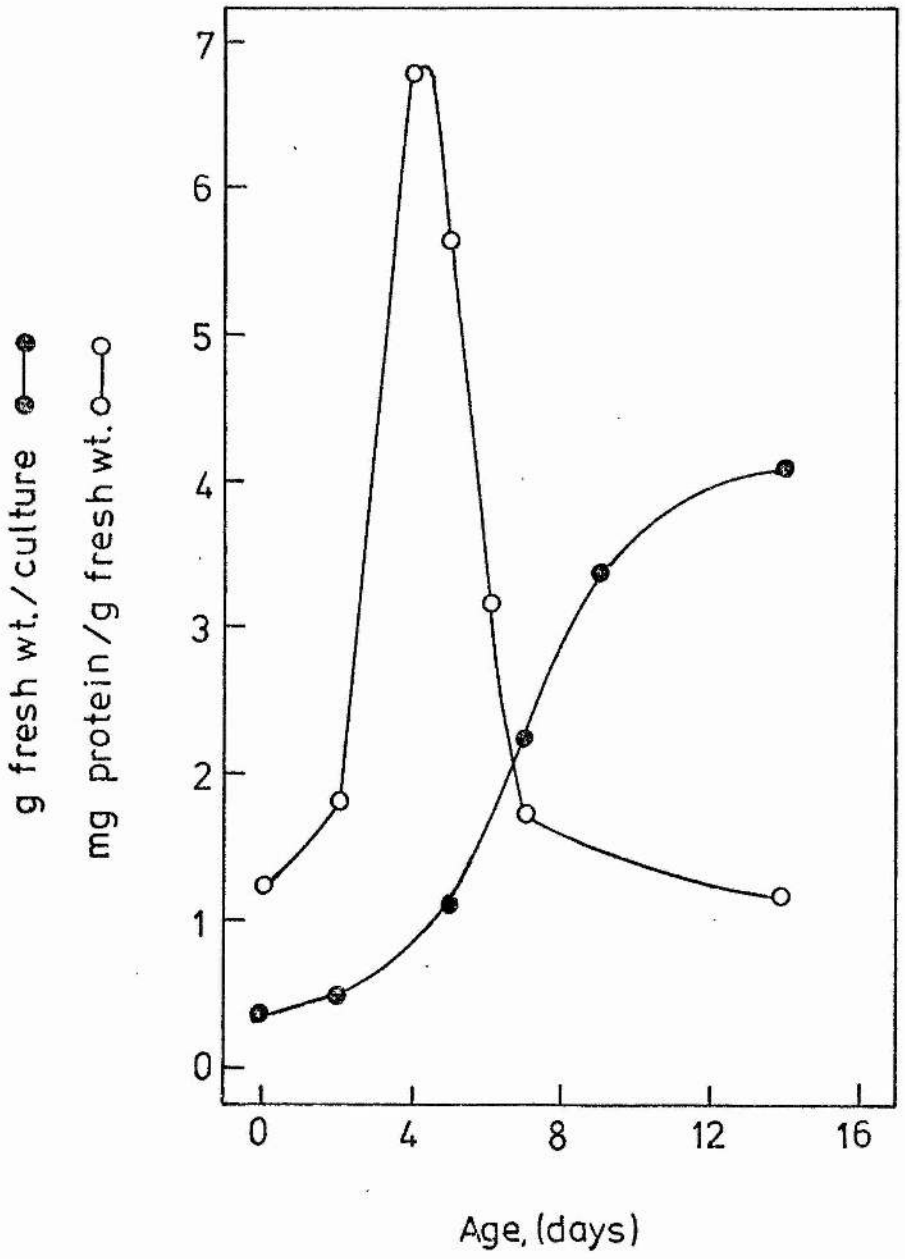


Fig. 13 Changes in fresh weight and protein levels with culture age.

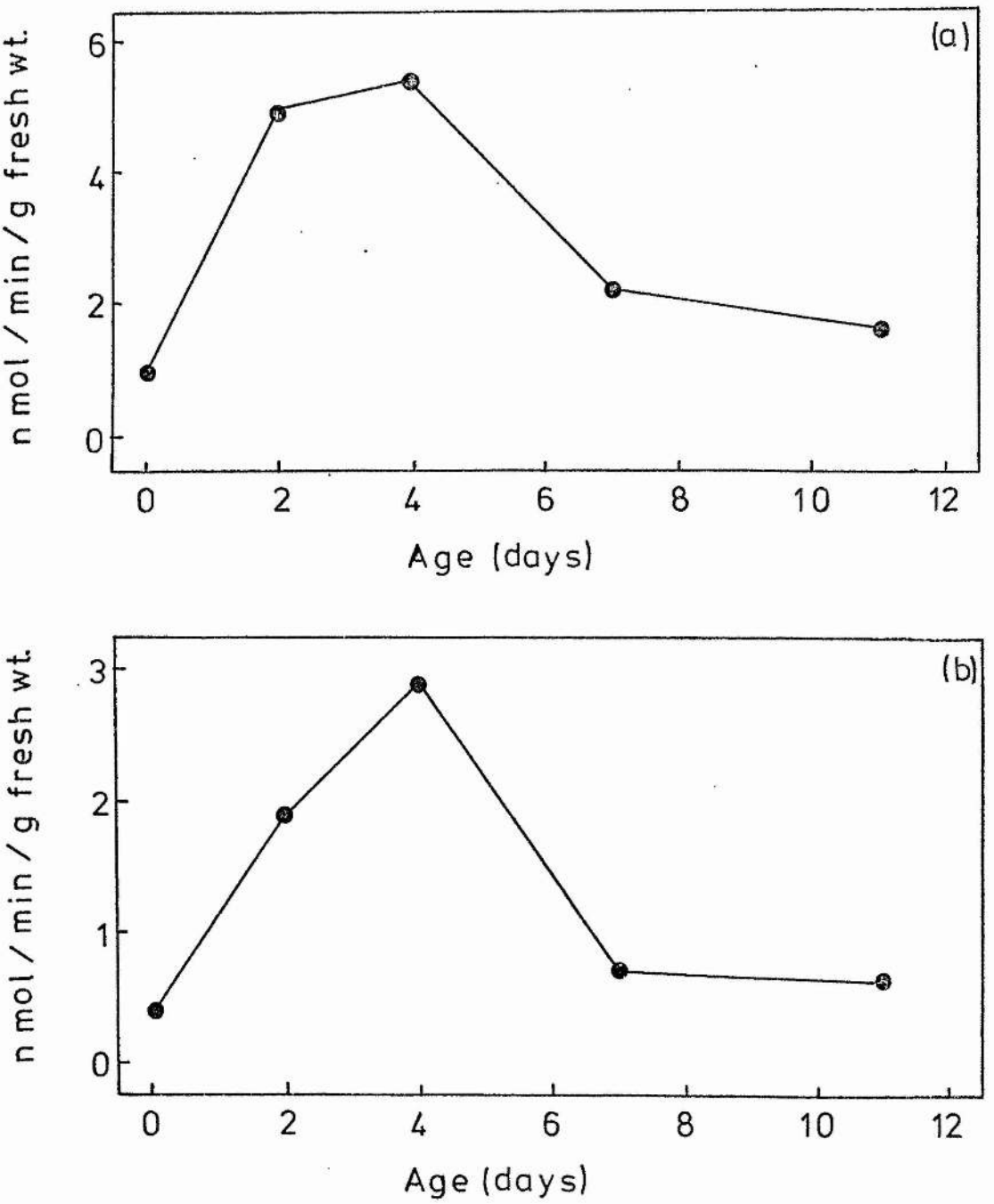


Fig. 14 Development of (a) arginine: tRNA ligase activity and of (b) leucine: tRNA ligase activity during growth of tobacco XD cells in M-1D medium.

Alteration of the Amino Acid Pool

(a) Transfer of cells into nitrateless medium

When stationary phase tobacco XD cells previously grown in M-1D are transferred into nitrateless medium they do not grow but remain viable, since growth can be restarted by adding nitrate even as late as 7 days after subculture. The amino acid pool is also depleted, being only 10-15% of that of cells grown in M-1D medium (Wray & Brice, unpublished work). Under these conditions therefore the activities of amino acid: tRNA ligases might be expected to be derepressed in a similar manner to that found in rat liver (Mariani et al., 1963) so that the fraction of amino acid involved in protein synthesis is increased.

To test this, stationary phase XD cells were transferred into nitrateless M-1D medium and the activity of leucine: and arginine: tRNA ligases followed (Fig. 15). As can be seen the activities of both enzymes remain fairly constant after transfer, unlike the situation in normal M-1D where a 7 fold increase in enzyme activity is observed. This result is therefore in contrast to rat liver where starvation results in an increase in the activity of amino acid: tRNA ligases (Mariani et al., 1963), and also to bacteria where restriction of the supply of a particular amino acid specifically increases the rate of synthesis of its cognate ligase (Nass & Neidhardt, 1967; McGinnis & Williams, 1971; Gahr & Nass, 1972). However it might be that under conditions of nitrate restriction where all amino acids are restricted there is a general run down of the cell's biosynthetic apparatus. This may prevent any derepression of arginine:and leucine: tRNA ligases. In certain micro-organisms for example restriction of one amino acid may bring about the 'stringent response' which involves a reduction in the rate of several biochemical processess including RNA synthesis, nucleotide synthesis and protein turnover (Neidhardt,

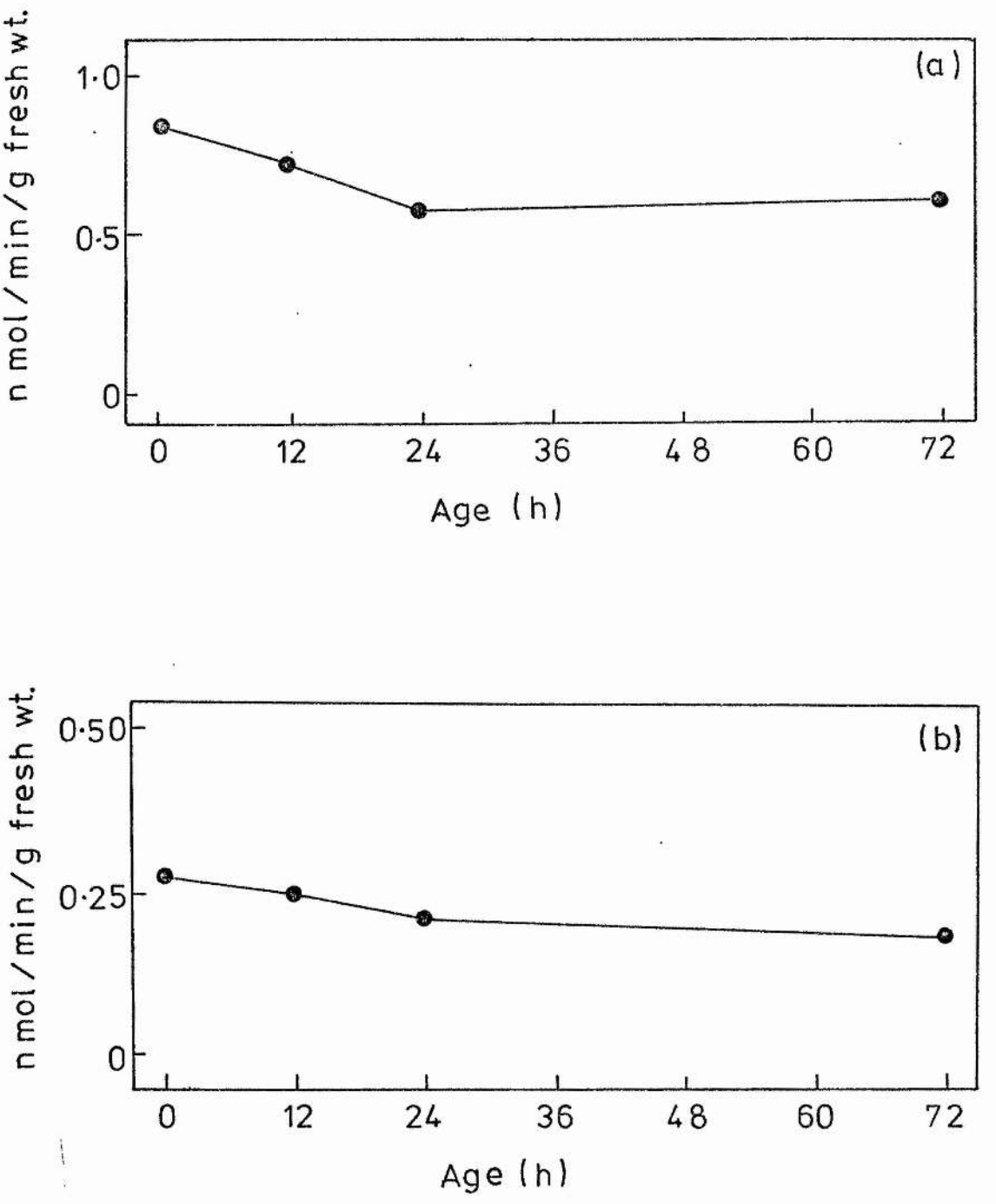


Fig. 15 Change in (a) arginine: tRNA ligase activity and in (b) leucine: tRNA ligase activity during growth of tobacco XD cells in nitrateless M-1D medium.

1966; Gallant et al., 1970; Haseltine & Block, 1973).

Another approach which could be used to deplete the amino acid pool would be to isolate mutants auxotrophic for a particular amino acid. This offers the great advantage of being able to restrict one specific amino acid. However since mutations conferring auxotrophy are recessive, their isolation needs to be carried out on haploid material and with the advent of anther (Nitsch & Nitsch, 1969) and pollen (Nitsch, 1974) culture techniques this has now become possible.

(b) Removal of arginine from the culture medium of cells grown in the presence of arginine.

Most single amino acids inhibit cell growth due to an effect on the nitrate assimilation pathway (Filner, 1966). Arginine however does not have this property allowing its effect on arginine: tRNA ligase activity to be studied.

In a preliminary experiment to study the effect of arginine on the growth of tobacco XD cells, stationary phase cells were transferred into M-1D medium supplemented either with 10^{-3}M or 10^{-4}M arginine (Fig. 16). It was found that while growth was stimulated by 10^{-4}M arginine, it was slightly inhibited by 10^{-3}M arginine. In addition cells grown in 10^{-3}M arginine did not clump together and were brown in appearance which contrasts with the clumpy nature and pale yellow colour of M-1D cells. It would appear therefore that 10^{-3}M arginine has some adverse effect on cell growth which might interfere with the development of arginine: tRNA ligase in a way quite unrelated to changes in the arginine levels of the amino acid pool. Thus in these studies 10^{-4}M arginine was used to supplement M-1D medium.

Experiments performed by Wray & Brice (unpublished work) indicated that there was no repression or derepression of arginine: tRNA ligase activity when stationary phase M-1D cells were transfered

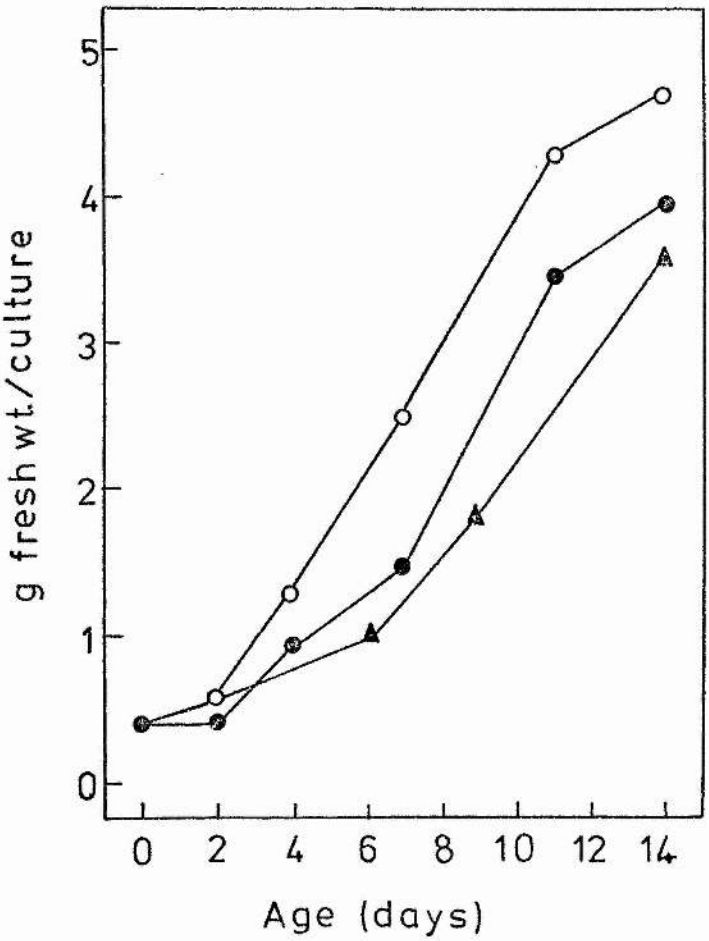


Fig. 16 Changes in fresh weight with culture age of cells grown in:-

M-1D

M-1D + 10^{-4}M arginine

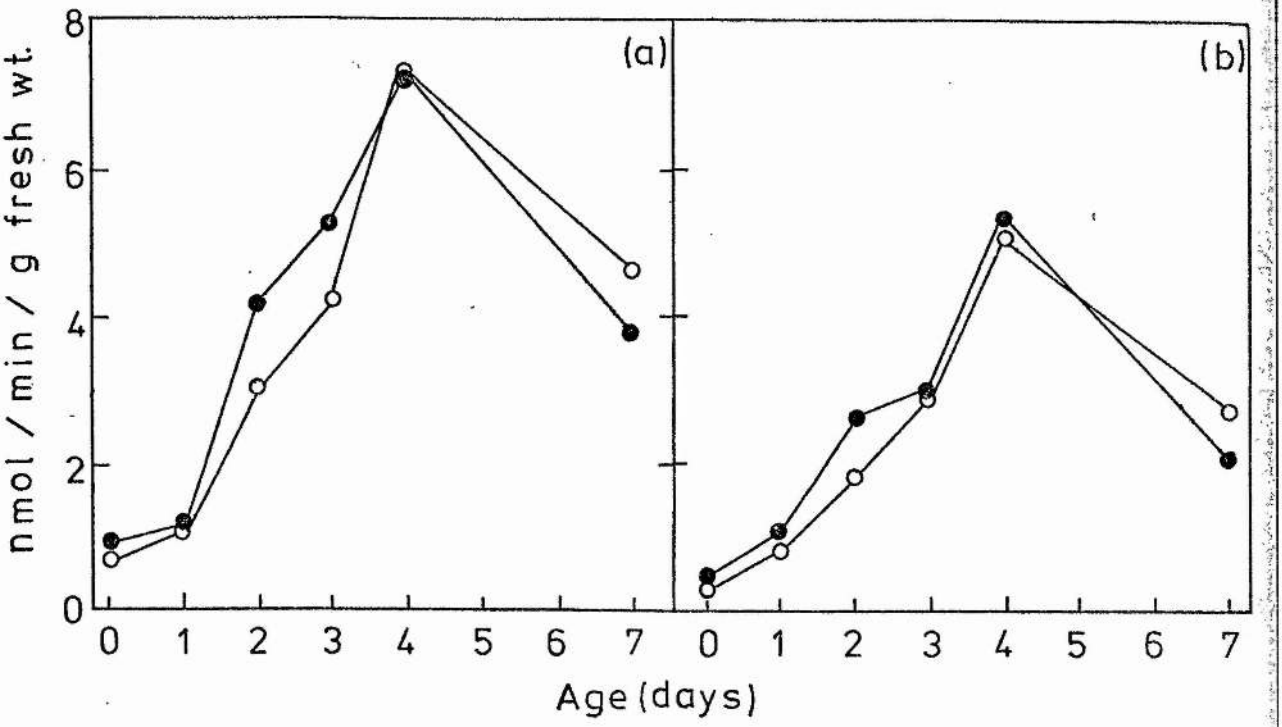
M-1D + 10^{-3}M arginine

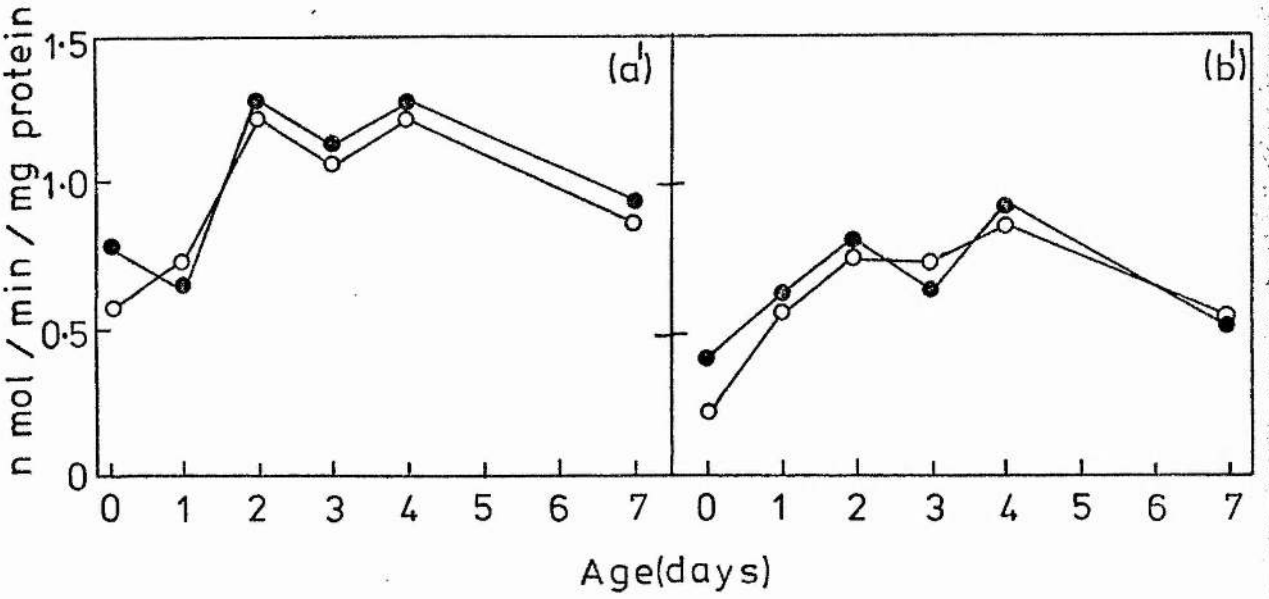
to M-1D medium supplemented with 10^{-4}M arginine. Ehresmann et al. (1971), however, have reported that removal of valine from the culture medium of yeast cells grown in the presence of valine causes a 2 fold increase in the specific activity of valine: tRNA ligase. To see if a similar effect could be demonstrated with arginine in tobacco XD cells, cells were grown for 50 generations in M-1D medium supplemented with 10^{-4}M arginine and following transfer of such stationary phase cells into normal M-1D medium, the development of protein levels and arginine: tRNA ligase activity was followed. As a control to ensure that any observed effect was specific, the development of leucine: tRNA ligase activity was also followed (Fig. 17). The data shows that both enzymes develop in an almost identical manner to enzyme extracted from M-1D cells and therefore there is no evidence for an increase in specific activity of arginine: tRNA ligase similar to that found for the valine enzyme in yeast (Ehresmann et al., 1971).

This is assuming that (1) exogenously supplied arginine is taken up by the cells and (2) a concentration of 10^{-4}Marginine is sufficient to maintain an increased intracellular level of amino acid over a 14 day growth period. The work of Widholm (1971) suggests that exogenously supplied amino acids do raise the intracellular levels but it is also possible that amino acid supplied exogenously is not equivalent to amino acid synthesised endogenously. The isolation of mutant cell lines which overproduce specific amino acids may help to solve some of these problems.

It therefore appears that altering the amino acid pool does not affect the development of arginine: and leucine: tRNA ligases, suggesting the amino acid level <u>per se</u> is not involved in the regulation of these enzymes.

Fig. 17 Changes in activity and specific activity of (a), (a') arginine: tRNA ligase and of (b), (b') leucine: tRNA ligase during growth of tobacco XD cells in M-1D ●——● and following transfer of stationary phase cells, previously grown for 50 generations in M-1D supplemented with 10^{-4}M arginine, into M-1D O——O


nmol / min / g fresh wt.
(a)
(b)
0
2
4
6
8
0 1 2 3 4 5 6 7
Age (days)


n mol / min / mg protein
(a')
(b')
0
0·5
1·0
1·5
0 1 2 3 4 5 6 7
Age(days)

Suitability of Certain Amino Acid Analogues for Lowering in Vivo Levels of Aminoacyl-tRNA

Although amino acids do not appear to be involved in the regulation of amino acid: tRNA ligases in tobacco XD cells, it may be that a change in the *in vivo* level of aminoacyl-tRNA is the signal that controls their synthesis and that although supplementation of medium with arginine leads to an increased arginine pool, there is no concomitant increase in the level of arginyl-$tRNA^{Arg}$. Such a role for aminoacyl-tRNA has been suggested in micro-organisms where histidyl-$tRNA^{His}$ has been implicated in the regulation of histidine: tRNA ligase in *Salmonella typhimurium* (McGinnis & Williams, 1972a,b).

In an attempt to lower the *in vivo* level of a particular aminoacyl-tRNA, three amino acid analogues, amitrole, leucine hydroxamate and canavanine were studied either as inhibitors of amino acid biosynthesis to reduce the amount of amino acid available for aminoacylation, or as competitive inhibitors of amino acid: tRNA ligases which whilst not being attached to tRNA themselves would prevent the attachment of the cognate amino acid. There are two important requisites for these analogues, firstly, they must not inhibit cell growth at any other site and secondly they must not be attached to tRNA, since long term experiments could not be performed if cells were dying due to incorporation of the analogue into their protein.

In all growth experiments involving analogues, stationary phase cells previously grown in M-1D were transfered into 250 ml flasks containing 100 ml of M-1D medium supplemented with the appropriate analogue or amino acid(s). At time intervals following this transfer, cells were harvested and fresh weights determined.

(1) Amitrole

The first analogue examined was a histidine analogue, 3-amino-

1,2,4-triazole (amitrole), which has been shown to inhibit the growth of yeast (Hilton, 1960), bacteria (Bond & Akers, 1961; Hilton et al., 1965) and many species of plants (Hilton et al., 1963; Davies, 1971).

In Paul's Scarlet rose cells (Davies, 1971) amitrole is thought to interfere with histidine biosynthesis by inhibiting immidazole-glycerolphosphate dehydratase and similar observations have been made in a number of micro-organisms (Klopotowski & Wiater, 1965; Hilton et al., 1965).

As a first step, to determine whether amitrole exhibited a similar effect on tobacco XD cells, stationary phase cells were transferred to M-1D medium supplemented with different concentrations of amitrole. The growth curves so obtained (Fig. 18) indicated amitrole was very toxic to growth at a concentration of 10^{-3}M. This was confirmed when addition of 10^{-3}M amitrole to exponentially growing cells caused a 45% growth inhibition after 10 days (Fig. 19).

Since in both micro-organisms and plants, amitrole is thought to interfere with histidine biosynthesis, it might be expected that the growth inhibition observed with tobacco cells should be reversed by the addition of histidine to the growth medium. Unfortunately the situation is complicated because histidine itself inhibits the growth of tobacco XD cells (Filner, 1966), although this is arginine reversible. Cells were therefore grown in M-1D medium containing amitrole and various concentrations of histidine and/or arginine to see if the amitrole inhibition could be reversed (Fig. 20). Although histidine and arginine together slightly reversed the amitrole inhibition so also did 10^{-4}M arginine alone, suggesting that this reversible inhibition was in response to arginine rather than histidine.

These results are consistent with the findings from other plant systems where histidine fails to reverse amitrole mediated growth

Fig. 18 Effect of supplementing M-1D with amitrole on growth of tobacco XD cells.

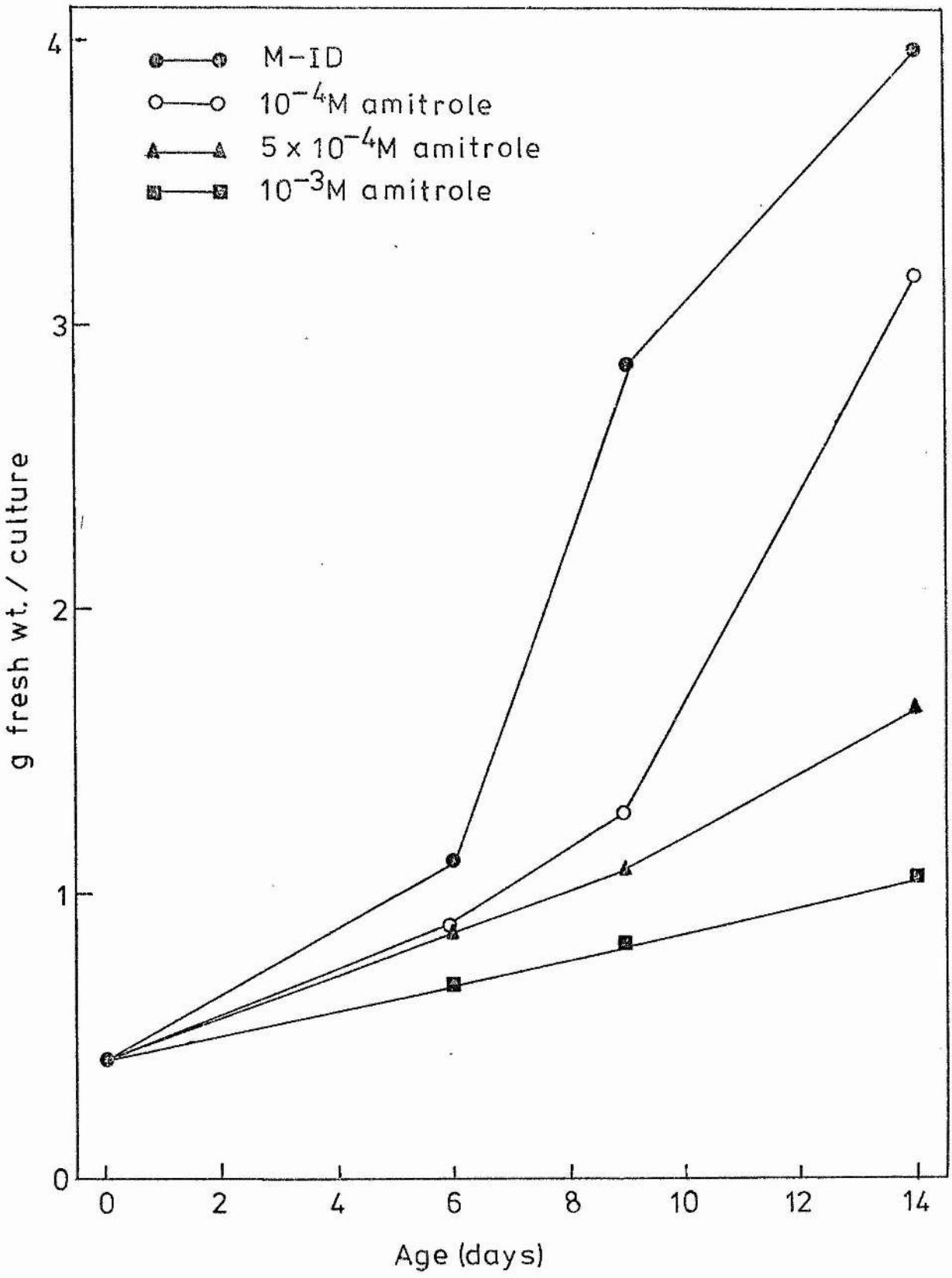

M-ID
10⁻⁴M amitrole
5 x 10⁻⁴M amitrole
10⁻³M amitrole
g fresh wt. / culture
0
1
2
3
4
0
2
4
6
8
10
12
14
Age (days)

Fig. 19 Effect of adding amitrole to a concentration of 10^{-3}M on exponentially growing tobacco XD cells.

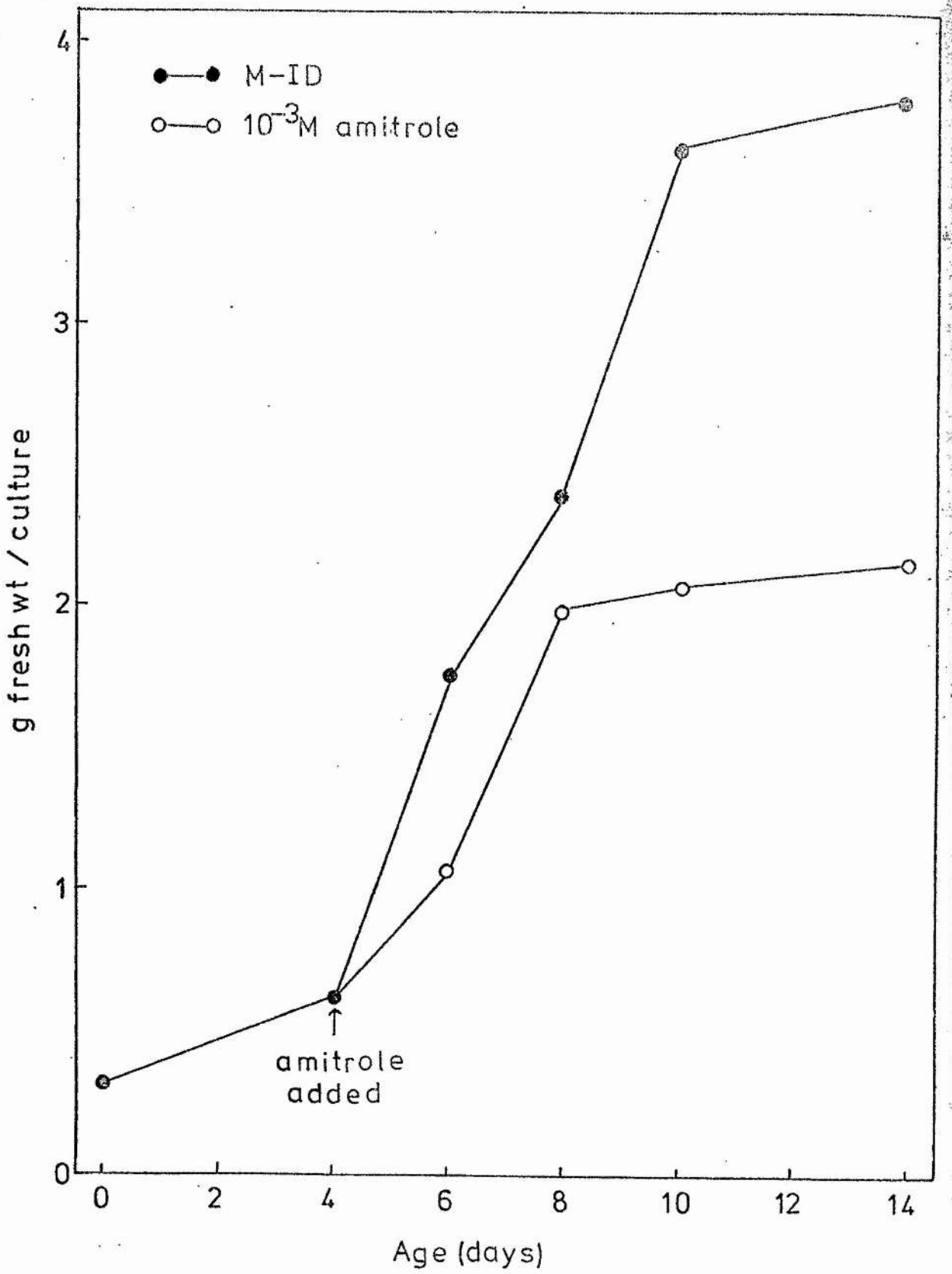
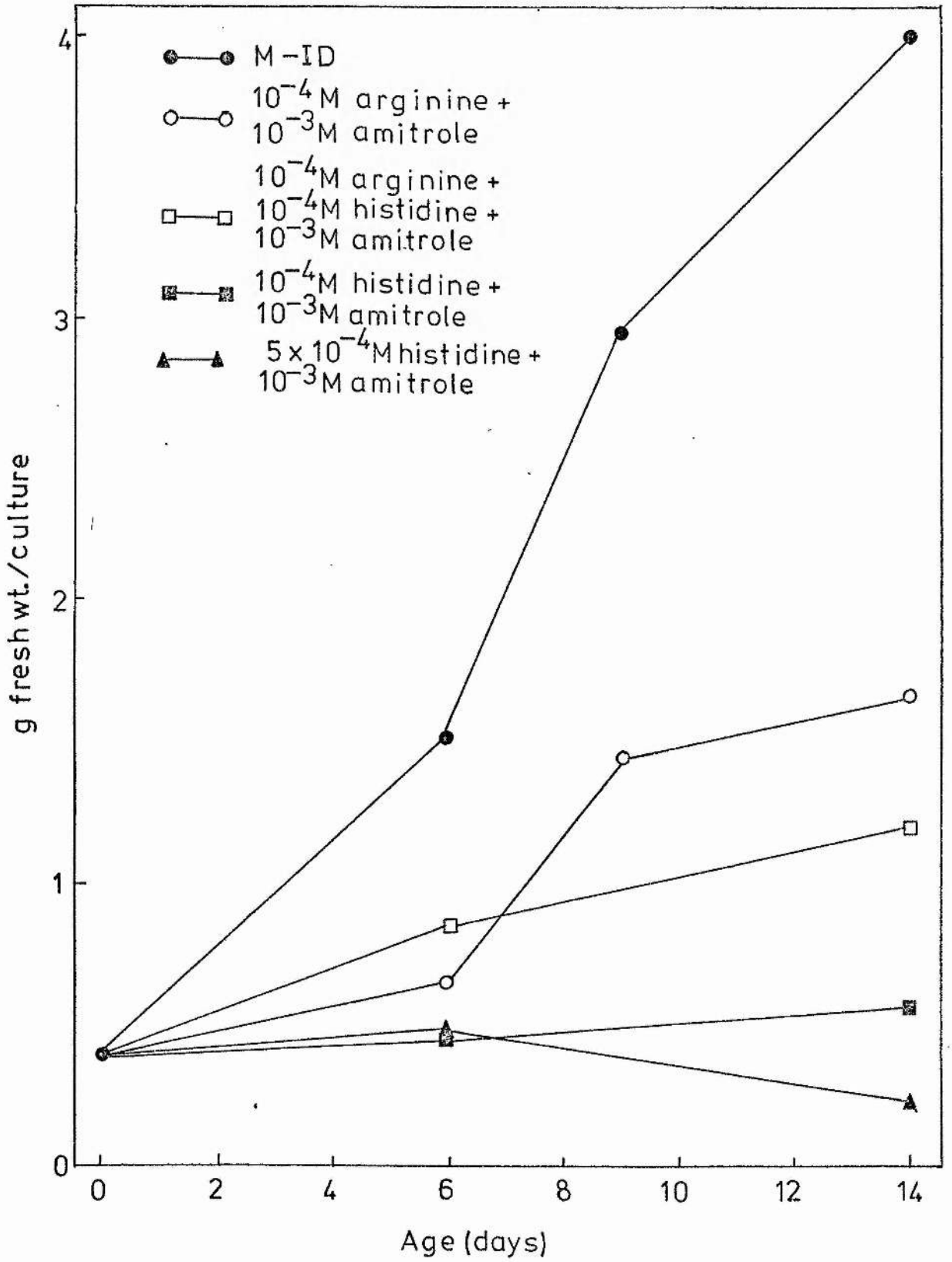

M-ID
10⁻³M amitrole
g fresh wt / culture
0
1
2
3
4
amitrole added
0
2
4
6
8
10
12
14
Age (days)

Fig. 20 Effect of arginine and histidine on the amitrole mediated growth inhibition of tobacco XD cells.


M-ID
10⁻⁴M arginine + 10⁻³M amitrole
10⁻⁴M arginine + 10⁻⁴M histidine + 10⁻³M amitrole
10⁻⁴M histidine + 10⁻³M amitrole
5 x 10⁻⁴M histidine + 10⁻³M amitrole
g fresh wt./culture
Age (days)
0
1
2
3
4
0
2
4
6
8
10
12
14

inhibition (Hilton, 1969). In yeast, however, histidine alone overcomes the toxicity of amitrole (Weyter & Broquist, 1960) but in micro-organisms a mixture of histidine and adenine are found to be better than either alone (Weyter & Broquist, 1960; Hilton et al., 1965). This suggests that in addition to interfering with histidine biosynthesis amitrole may also affect purine biosynthesis. If this were true for tobacco XD cells, amitrole would clearly be unsuitable for our purposes since it would be impossible to tell whether changes in the in vivo levels of histidine: tRNA ligase were a consequence of an alteration in the level of histidyl-tRNA or the action of amitrole on purine biosynthesis.

To investigate the possibility that amitrole might affect purine biosynthesis, cells were transferred into M-1D medium supplemented with different concentrations of adenine (Fig. 21). Adenine initially inhibits cell growth and the higher the concentration, the longer the cells require to overcome the inhibitory effect.

When cells were transferred into M-1D medium supplemented with both adenine and amitrole (Fig. 22), the growth curves obtained suggested that adenine did reverse the amitrole inhibition to some extent, but the results were far from conclusive.

Although the data from these experiments do no allow us to conclude whether amitrole toxicity is mediated via either histidine or purine biosynthesis, the results from a wide variety of other organisms (Hilton, 1969; Davies, 1971), suggest amitrole may inhibit at more than one site and thus would be unsuitable for lowering the in vivo levels of histidyl-tRNA in tobacco XD cells.

Amitrole toxicity could also be mediated at the level of histidine: tRNA ligase. To investigate this, the rate of aminoacylation of tRNA by this enzyme was determined both in the presence and absence of 10^{-3}M amitrole(Fig.23). The data shows that amitrole had no effect on

Fig. 21 Effect of supplementing M-1D with adenine on the growth of tobacco XD cells.

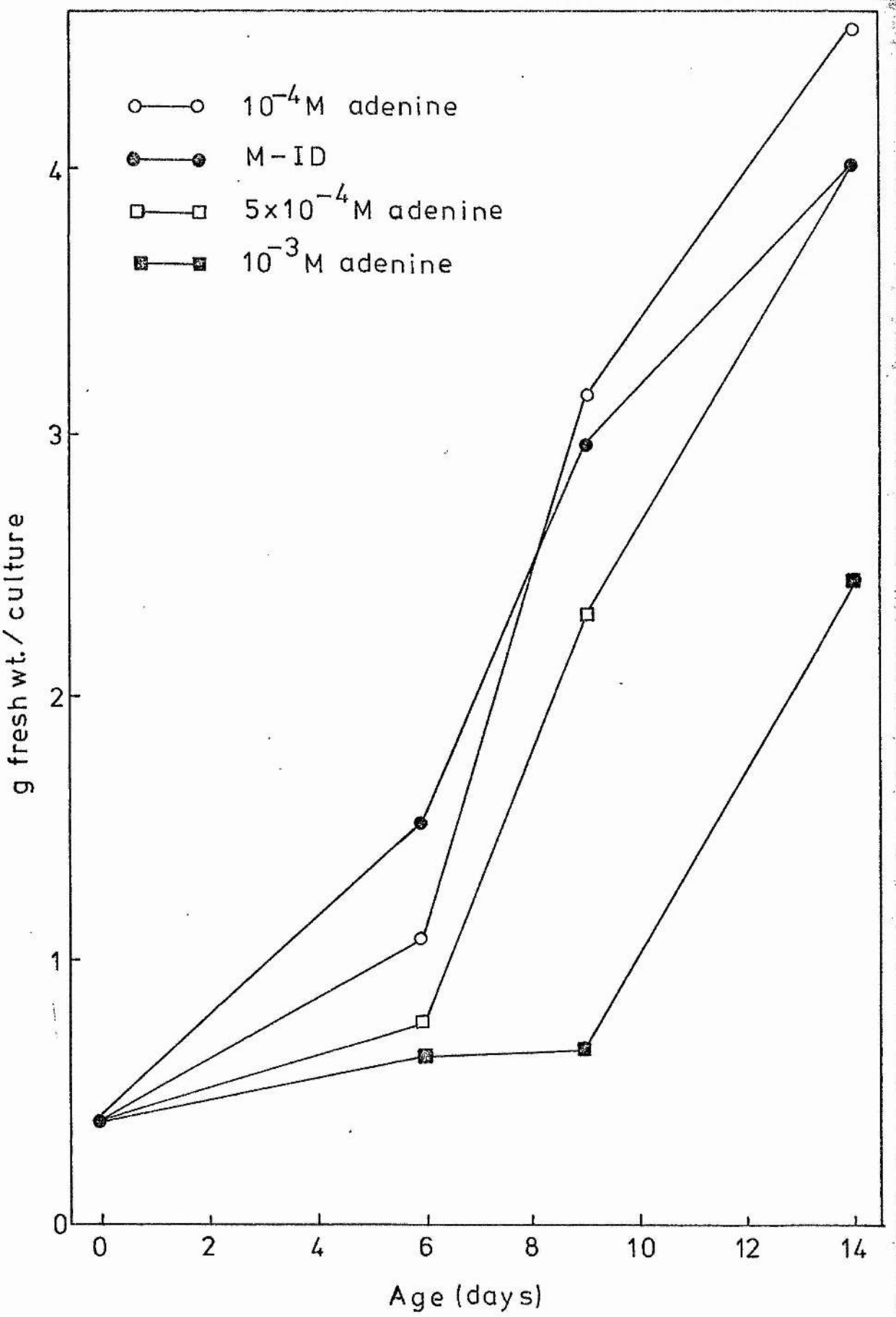

10⁻⁴M adenine
M-ID
5x10⁻⁴M adenine
10⁻³M adenine
g fresh wt./culture
0
1
2
3
4
0
2
4
6
8
10
12
14
Age (days)

Fig. 22 Effect of adenine on the amitrole mediated growth inhibition of tobacco XD cells.

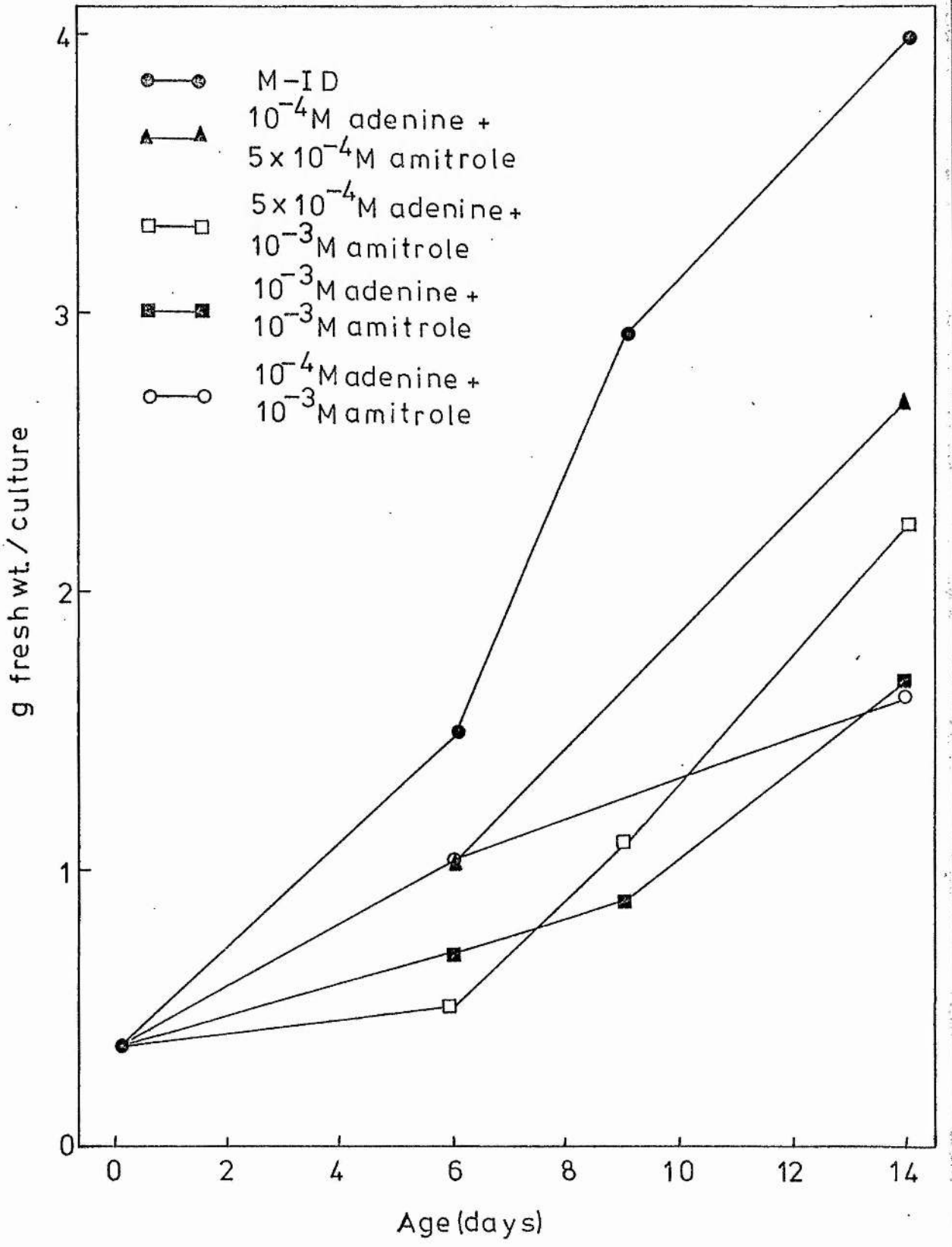
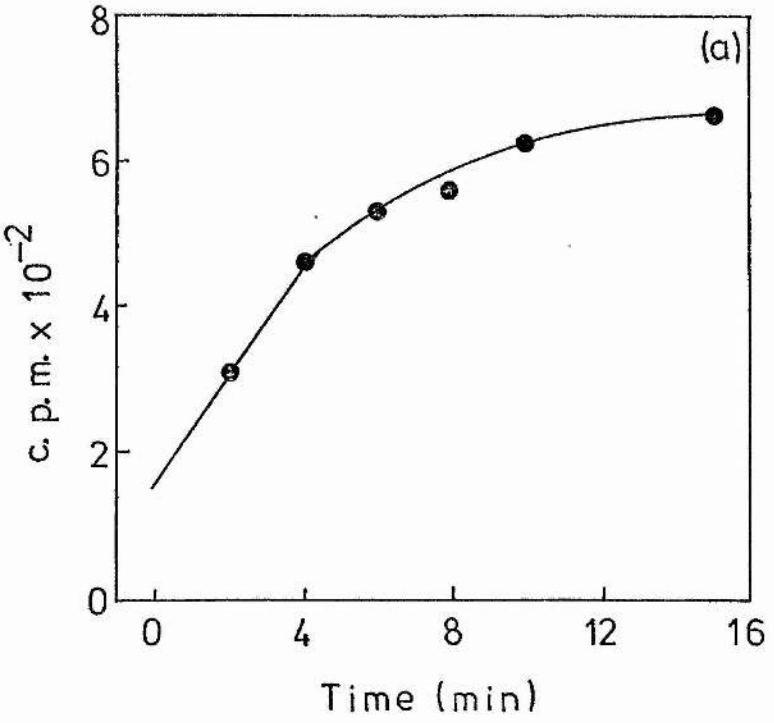
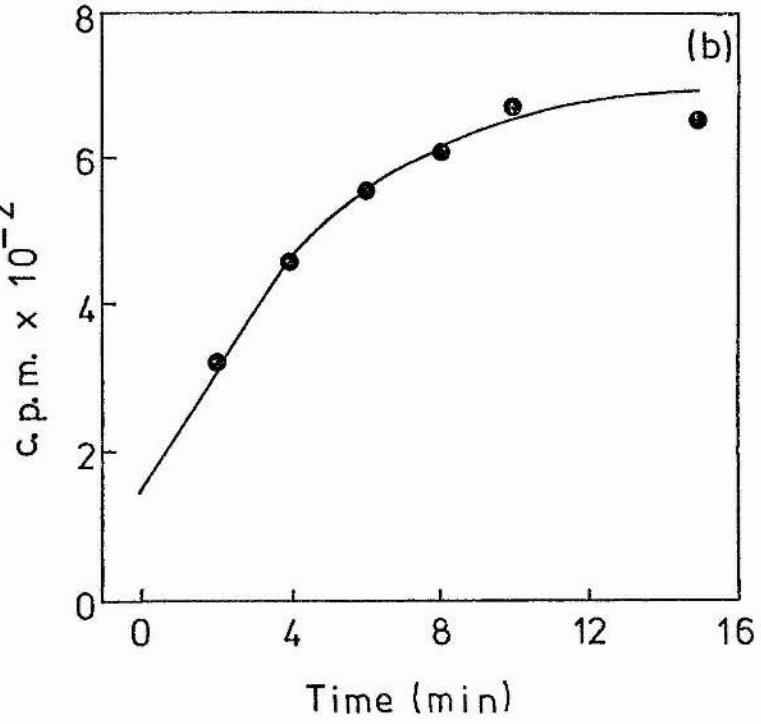

M-ID
10⁻⁴M adenine + 5x10⁻⁴M amitrole
5x10⁻⁴M adenine + 10⁻³M amitrole
10⁻³M adenine + 10⁻³M amitrole
10⁻⁴M adenine + 10⁻³M amitrole
g fresh wt./culture
Age (days)
0
1
2
3
4
0
2
4
6
8
10
12
14

Fig. 23 Kinetics of aminoacylation of tRNA with [$^{-3}$H]- histidine in (a) the absence of and (b) the presence of 10^{-3}M amitrole. The reaction mixture, as described in the materials and methods section, contained 1μmol Mg^{2+}, 0.2μmol ATP and 120μg tRNA.


(a)
8
6
4
2
0
c.p.m. x 10^{-2}
0
4
8
12
16
Time (min)


(b)
8
6
4
2
0
c.p.m. x 10^{-2}
0
4
8
12
16
Time (min)

the rate of aminoacylation of tRNA, suggesting growth inhibition was not a consequence of reduced protein synthesis caused by an inhibition of histidine: tRNA ligase or of incorporation of amitrole into cell protein.

In conclusion, the mechanism(s) whereby amitrole causes growth inhibition of tobacco XD cells is unclear and as well as those mechanisms we have investigated, the analogue could also inhibit specific membrane transport systems for histidine or be degraded into toxic substances. However, the results from our studies clearly indicate that amitrole cannot be used to lower the in vivo level of histidyl-tRNA.

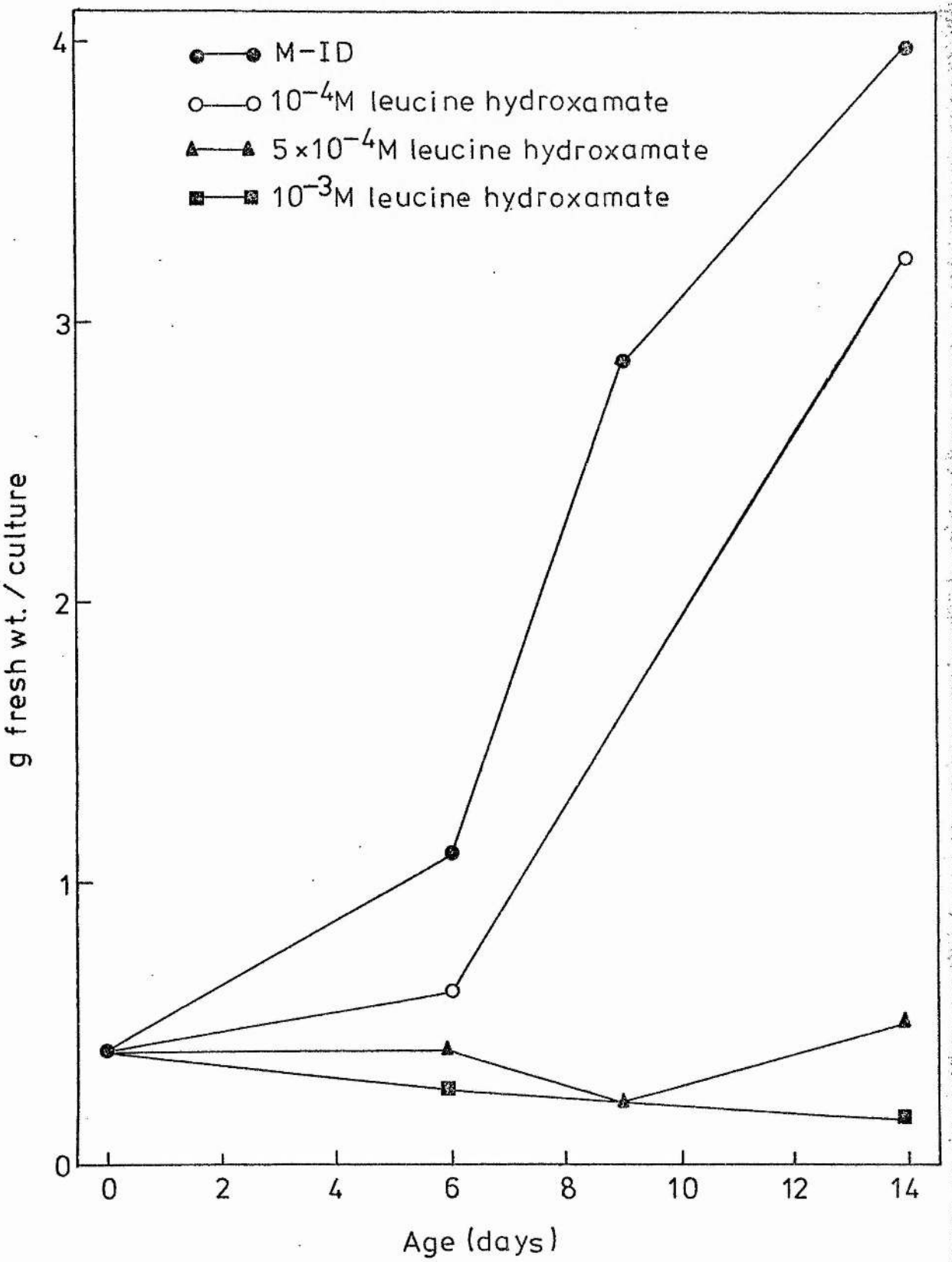
(2) Leucine hydroxamate

Tosa & Pizer (1971a,b) reported that serine hydroxamate inhibits the growth of E. coli and is also a competitive inhibitor of serine: tRNA ligase. Unfortunately tobacco XD cells possess very low levels of serine: tRNA ligase and so it was decided to investigate the properties of a similar analogue, leucine hydroxamate.

Transfer of stationary phase cells into fresh M-1D medium containing 10^{-4}M leucine hydroxamate resulted in a 20% inhibition of growth after 14 days, whereas 10^{-3}M leucine hydroxamate completely prevented cell growth and turned the cells brown (Fig. 24). To ascertain whether the inhibition caused by this analogue was related to leucine metabolism, the ability of leucine to relieve the inhibition was tested. Unfortunately leucine is similar to histidine in that it causes an arginine reversible inhibition of cell growth. However, even when cells were transfered into M-1D medium containing 10^{-3}M leucine hydroxamate, 10^{-3}M leucine and 10^{-4}M arginine no growth was observed. Therefore leucine could not reverse the leucine hydroxamate growth inhibition.

To determine if leucine hydroxamate inhibited leucine: tRNA

Fig. 24 Effect of supplementing M-1D with leucine hydroxamate on the growth of tobacco XD cells.


M-ID
10⁻⁴M leucine hydroxamate
5×10⁻⁴M leucine hydroxamate
10⁻³M leucine hydroxamate
g fresh wt. / culture
Age (days)
0
1
2
3
4
0
2
4
6
8
10
12
14

ligase the initial rates of aminoacylation of tRNA with [^{3}H]-leucine were determined in the presence of 10^{-4} and 3×10^{-4}M leucine hydroxamate (Fig. 25a). At 3×10^{-4}M a 50% inhibition of enzyme activity was observed.

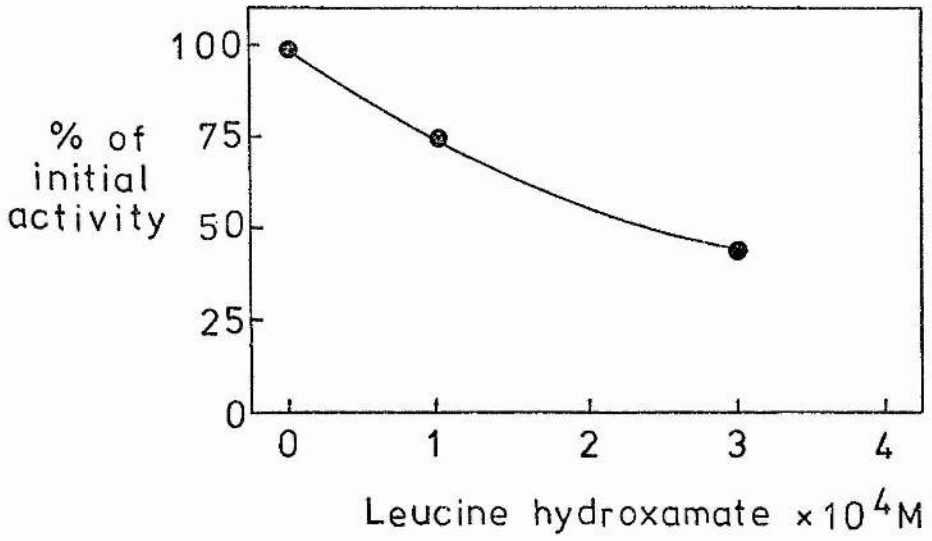
Having established that leucine hydroxamate did inhibit leucine: tRNA ligase activity, the nature of this inhibition was investigated. The initial rates of aminoacylation with [^{3}H]-leucine were determined at amino acid concentrations of 1 μM, 2.5 μM, 5.0 μM and 10 μM both in the presence and absence of 300 μM leucine hydroxamate. Lineweaver-Burk plots of the data (Fig. 25b) suggested that leucine hydroxamate was a competitive inhibitor of leucine: tRNA ligase. The Km for leucine obtained from these studies was 7.1 μM, slightly less than the 8.3 μM found in the previous section. The Ki for leucine hydroxamate was 167 μM, suggesting that leucine: tRNA ligase has a poor affinity for the analogue in comparison to its natural substrate. This is in contrast to what is found in E. coli where serine: tRNA ligase has a higher affinity for the analogue than it does for its natural substrate (Tosa & Pizer, 1971b).

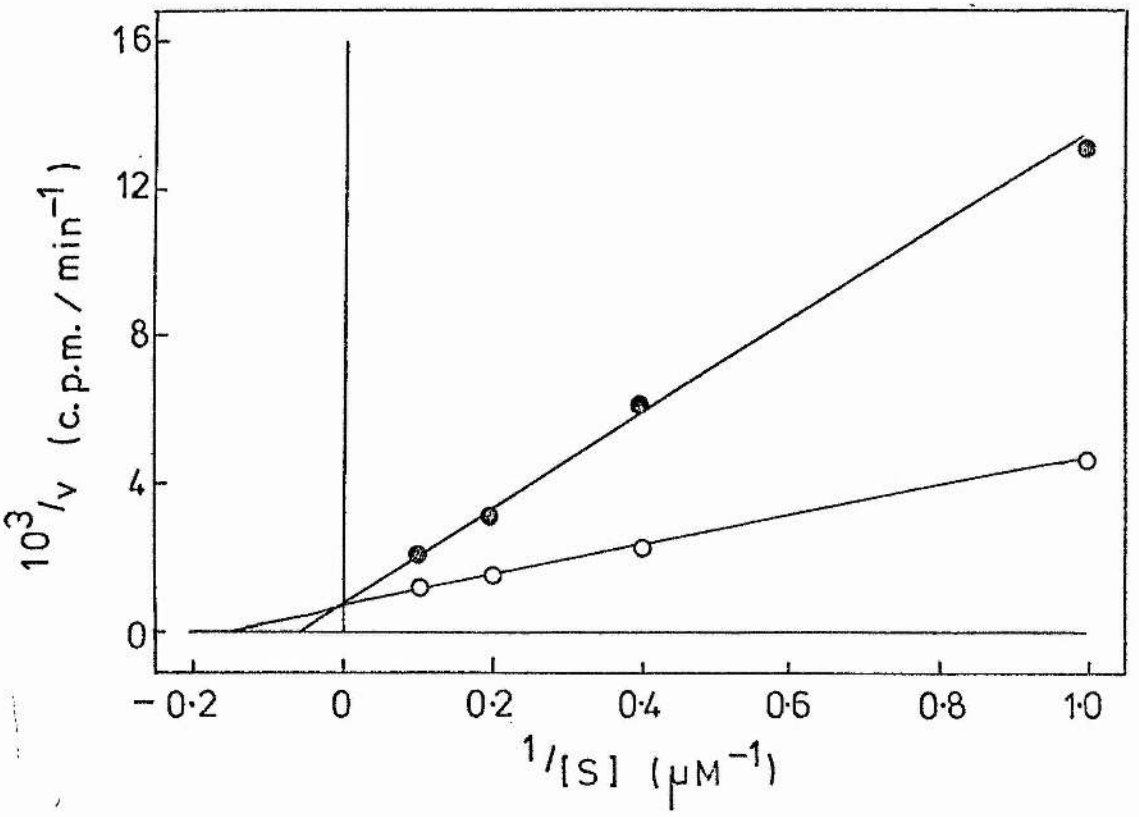
Because leucine hydroxamate is a competitive inhibitor of aminoacylation, its effectiveness in stopping growth depends on the concentration within the cell of both leucine and leucine hydroxamate and the relative affinities of these two compounds for the activating enzyme. However, since the affinity of leucine: tRNA ligase for leucine hydroxamate is so poor when compared with leucine, we wondered if addition of 10^{-3}M leucine hydroxamate to M-1D medium would produce a sufficiently high intracellular concentration to totally inhibit growth via inhibition of the leucine enzyme alone.

It is known that hydroxamic acids are usually heat labile and since leucine hydroxamate is autoclaved with M-1D medium at 20 lb psi the possibility that at least some of the inhibition was a result

Fig. 25a Inhibition of leucine: tRNA ligase by leucine hydroxamate.

Fig. 25b Determination of the Ki for leucine hydroxamate from Lineweaver-Burk plots of the initial rates of aminoacylation of tRNA with [^{3}H]-leucine in the presence of 300μM leucine hydroxamate ●——● and in the absence of 300μM leucine hydroxamate O——O


100
75
50
25
0
% of initial activity
0
1
2
3
4
Leucine hydroxamate ×10⁴M


16
12
8
4
0
10³/v (c.p.m./min⁻¹)
−0·2
0
0·2
0·4
0·6
0·8
1·0
1/[S] (μM⁻¹)

of a toxic breakdown product of leucine hydroxamate was investigated.

50 µg samples of leucine hydroxamate were spotted onto two sheets of Whatman No. 1 paper both before and after autoclaving and run in a butanol: acetic acid: water solvent for 12h. The chromatograms were sprayed either with ninhydrin to detect amino acids or with a solution of ferric chloride to detect hydroxamic acids, as described in the materials & methods section. The results suggest that some change in leucine hydroxamate occurs during autoclaving since the amount of the analogue appears to decrease (Fig. 26a). In addition, after autoclaving there is an increase in the intensity of the purple spot (Fig. 26b), presumed to be leucine, indicating that leucine hydroxamate might break down into leucine and perhaps a toxic amine product.

Taken together with the observations that leucine hydroxamate inhibition is not reversed by leucine and that leucine: tRNA ligase has a poor affinity for the analogue, it was concluded that a concentration of 10^{-3}M leucine hydroxamate was unlikely to result in a total growth inhibition simply by inhibiting leucine: tRNA ligase. The use of this analogue therefore to lower *in vivo* levels of leucyl-tRNA was unlikely to be successful.

(3) Canavanine

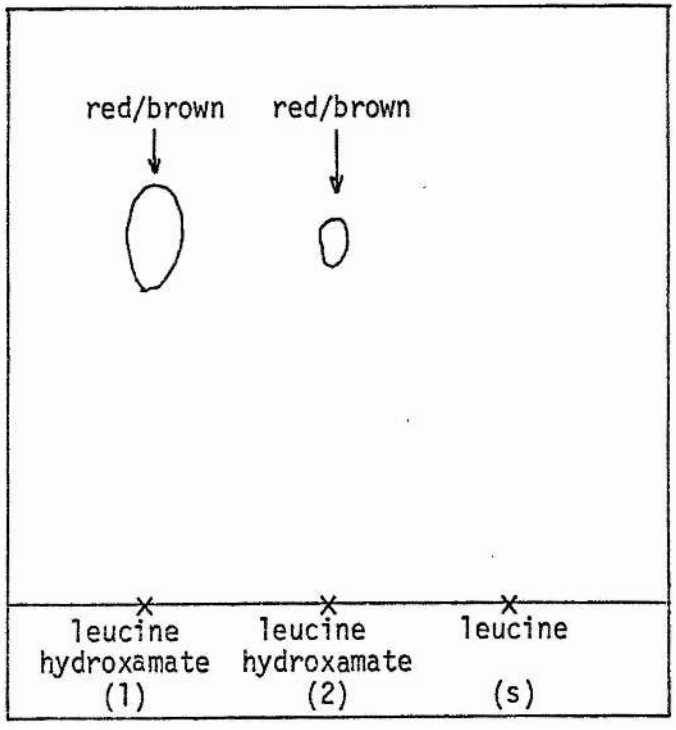
The final analogue investigated was the arginine analogue canavanine. At a concentration of 10^{-3}M it completely inhibited cell growth but as it appears to be attached to tRNA by arginine: tRNA ligase (Wray, unpublished work), it was not suitable for lowering the *in vivo* levels of tRNA.

In these studies therefore, the search for an amino acid analogue to lower *in vivo* levels of aminoacyl-tRNA were unsuccessful, but other analogues are currently being screened in this laboratory.

Fig. 26 Paper chromatography to show the effect of autoclaving at 20 lb psi on the composition of leucine hydroxamate. Chromatograms were sprayed (a) with ferric chloride to detect hydroxamate and (b) with ninhydrin to detect amino acids.

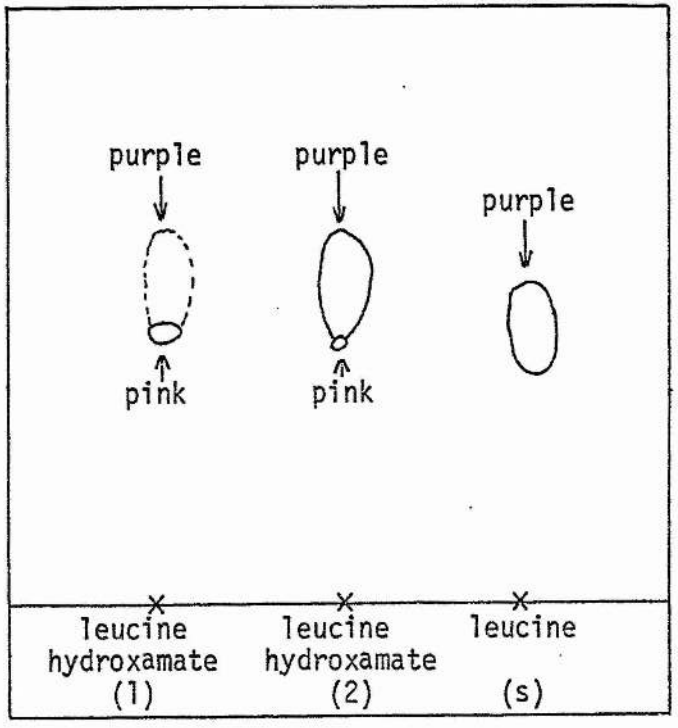
(a)

Ferric chloride sprayed



(b)

Ninhydrin sprayed



(1) before autoclaving (2) after autoclaving (s) leucine standard

SECTION 3

EVIDENCE FOR DE NOVO SYNTHESIS AND TURNOVER OF LEUCINE: AND ARGININE: tRNA LIGASES

INTRODUCTION

In this section we have employed the density labelling technique, as described in the general introduction, to look for evidence of de novo synthesis and turnover of arginine: and leucine: tRNA ligases, during the period that these enzymes increase in activity in growing tobacco XD cells (Fig. 14). In addition, we have looked for similar evidence when cells are placed under conditions of nitrate restriction, where it has been demonstrated that amino acid pools are depleted (Wray & Brice, unpublished work) and development of ligase activity is prevented (Fig. 15).

The density labelling technique was used in preference to the other methods available because it requires no enzyme purification. In contrast isotopic techniques may require repeated and laborious protein purifications to isolate newly synthesised labelled enzyme (Schimke, 1969; Parker et al., 1974). These procedures may inactivate unstable enzymes which is particularly pertinent to our studies, since we have shown that both leucine: and arginine: tRNA ligases are rather unstable (Fig. 10). Immunological techniques may also require a degree of enzyme purification and have the additional problem of raising antibodies (Kleinkopf et al., 1970; Peterson et al., 1973). There is also the possibility that any minor changes in enzyme structure which occur during synthesis might not be detected by the antibody reaction.

To show de novo synthesis and turnover in our studies, a buoyant density difference must be demonstrated between pre-existing protein, labelled by cell growth in heavy isotope, and protein which is newly formed in the absence of this heavy isotope.

Cell protein was labelled by growing XD cells in M-1D medium containing 2.5mM (^{-15}N) -nitrate as sole nitrogen source and 30% deuterium oxide (heavy medium). To ensure protein was fully labelled

with heavy isotope, cells were grown for at least 100 generations in heavy medium before density labelling experiments were performed.

A double label was employed since ^{15}N alone would only give a small density difference between labelled and unlabelled protein, and although deuterium oxide would produce the greatest density difference in comparison to other stable isotopes, in preliminary experiments levels of more than 30% were found to be inhibitory to cell growth.

Incorporation of ^{15}N into protein will be via the nitrate assimilation pathway (Fig. 1), whereas deuterium will be incorporated in metabolic reactions involving hydration such as in the citric acid cycle. The incorporation of deuterium in 1 turn of this cycle is shown in Fig. 27. It could be argued, however, that some deuterium might be incorporated by simple exchange with protons. Hvidt and Nielson (1966) have termed those protons which are bound to oxygen, sulphur or nitrogen atoms in a protein molecule as 'labile', since they will exchange fairly rapidly with protons in the surrounding medium. In contrast exchange of carbon bound protons is usually so slow as to be undetectable. 'Labile' protons in side groups with the exception of primary amide groups exchange very quickly (min) and much faster than 'labile' peptide and primary amide group protons. However, since the labelled enzyme is extracted and subjected to isopyenic equilibrium centrifugation for 65h in a totally proton environment before the buoyant density is determined, any labelling that might have occurred by exchange would be annulled.

Since the amino acid composition of these ligases is unknown the theoretical increase in density that would be expected by growing XD cells in heavy medium cannot be accurately calculated. However, if

FIG. 27 Incorporation of deuterium (D) into citric acid cycle intermediates via hydration reactions

(1) CITRATE SYNTHASE

(2a) ACONITASE

(2b) ACONITASE

(3a) ISOCITRATE DEHYDROGENASE

(3b) ISOCITRATE DEHYDROGENASE

(4) 2-OXOGLUTARATE DEHYDROGENASE

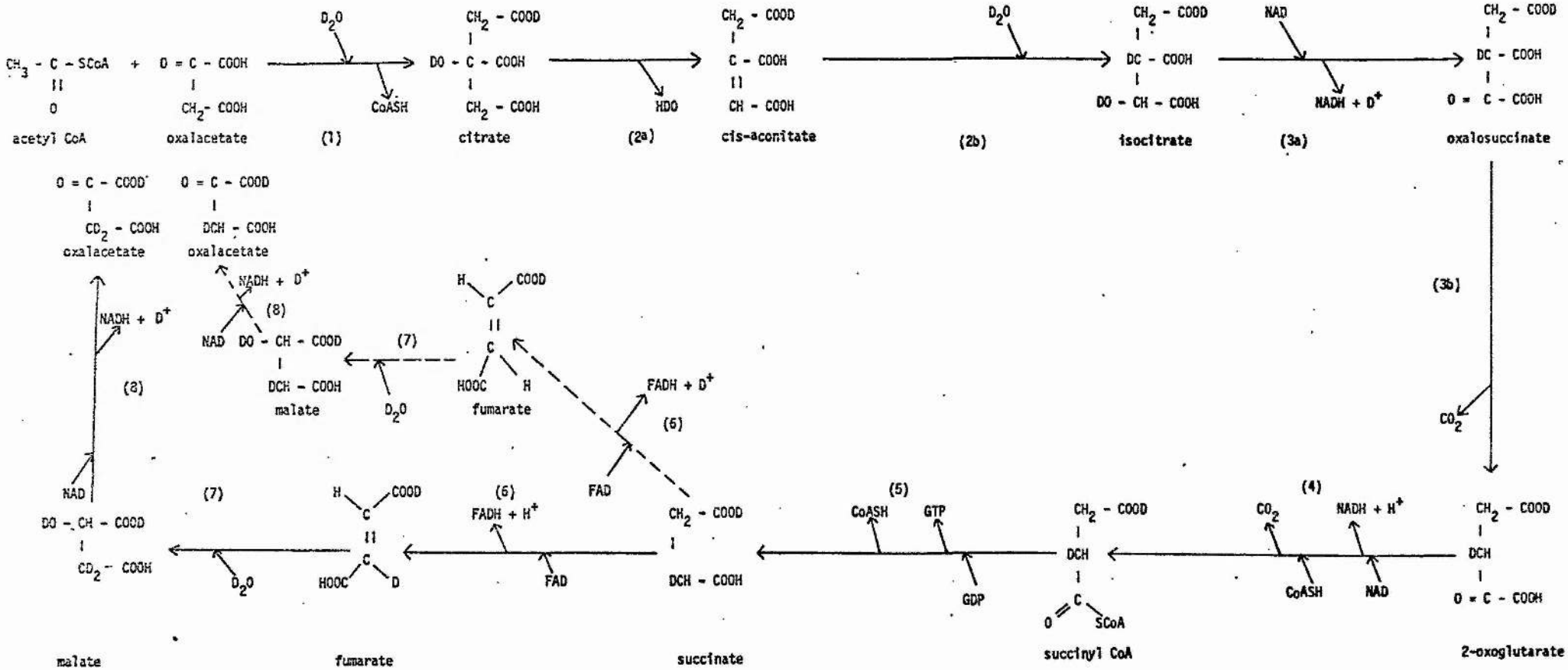
(5) SUCCINIC THIOKINASE

(6) SUCCINATE DEHYDROGENASE

(7) FUMARASE

(8) MALATE DEHYDROGENASE

Since carboxyl groups exist as COO^- only deuterium bound to carbon atoms will be incorporated into amino acids.


acetyl CoA
oxalacetate
(1)
citrate
(2a)
cis-aconitate
(2b)
isocitrate
(3a)
oxalosuccinate
(3b)
2-oxoglutarate
(4)
succinyl CoA
(5)
succinate
(6)
fumarate
(7)
malate
(8)
oxalacetate
oxalacetate
CoASH
HDO
NADH + D+
CO2
GTP
GDP
FAD
FADH + D+
FADH + H+
D2O
NAD

it is assumed that the average molecular weight of an amino acid in a protein is approximately 120, some estimate can be made.

Incorporation of ^{15}N would be expected to give an increase in density of about 0.8-1.0%, since the molecular weight of the amino acid would be increased by about 1 due to replacement of ^{14}N by ^{15}N in the α-amino nitrogen and amides of amino acids in the enzyme protein. Similarly, if this average amino acid contains 7 or 8 hydrogen atoms of which 30% have been replaced by deuterium, the resulting increase in molecular weight of about 2 would give an increase in density of about 1.5-1.8%. Thus the total increase in density that might be expected by double labelling cells in this way would be of the order of 2.5%.

RESULTS AND DISCUSSION

Comparison of the Aminoacylation and ATP-Pyrophosphate Exchange Assays in Detecting Leucine: tRNA Ligase Activity after Isopycnic Equilibrium Centrifugation.

In density labelling experiments the buoyant densities of leucine: and arginine: tRNA ligases would be determined from their distribution profiles following isopycnic equilibrium centrifugation in caesium chloride. Therefore, to determine which of the two assay techniques was most suitable for detecting ligase activity in density gradients, leucine: tRNA ligase was extracted from 5 day old tobacco XD cells and following centrifugation in caesium chloride the distribution of the enzyme was determined by either ATP-pyrophosphate exchange (Fig. 28a) or aminoacylation of tRNA (Fig. 28b).

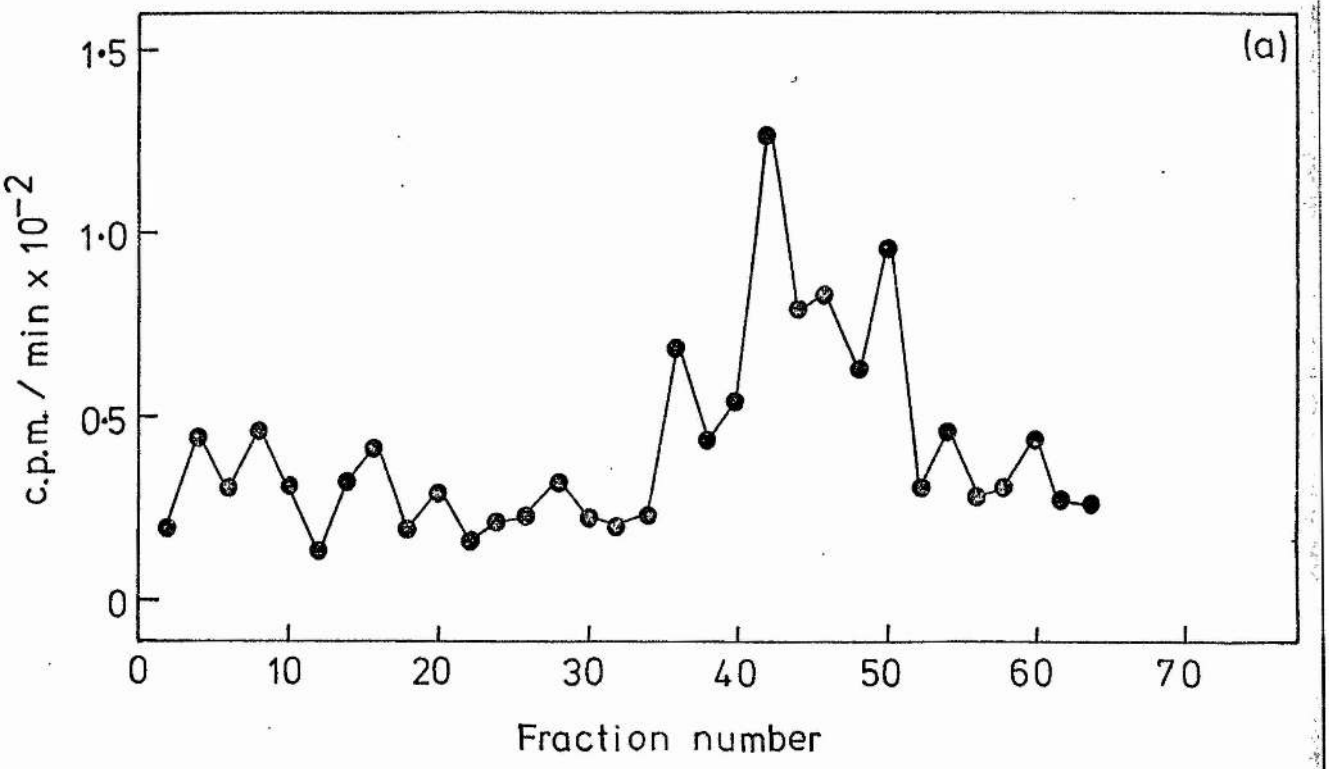
Several density gradients were performed using the ATP-pyrophosphate exchange assay with similar but not identical distribution patterns. These experiments demonstrate the poor reproducibility and high background levels which make the ATP-pyrophosphate exchange assay unsuitable for density labelling experiments. In contrast the aminoacylation assay gave a low constant background with a homogeneous Gaussian distribution and was therefore used in all subsequent experiments.

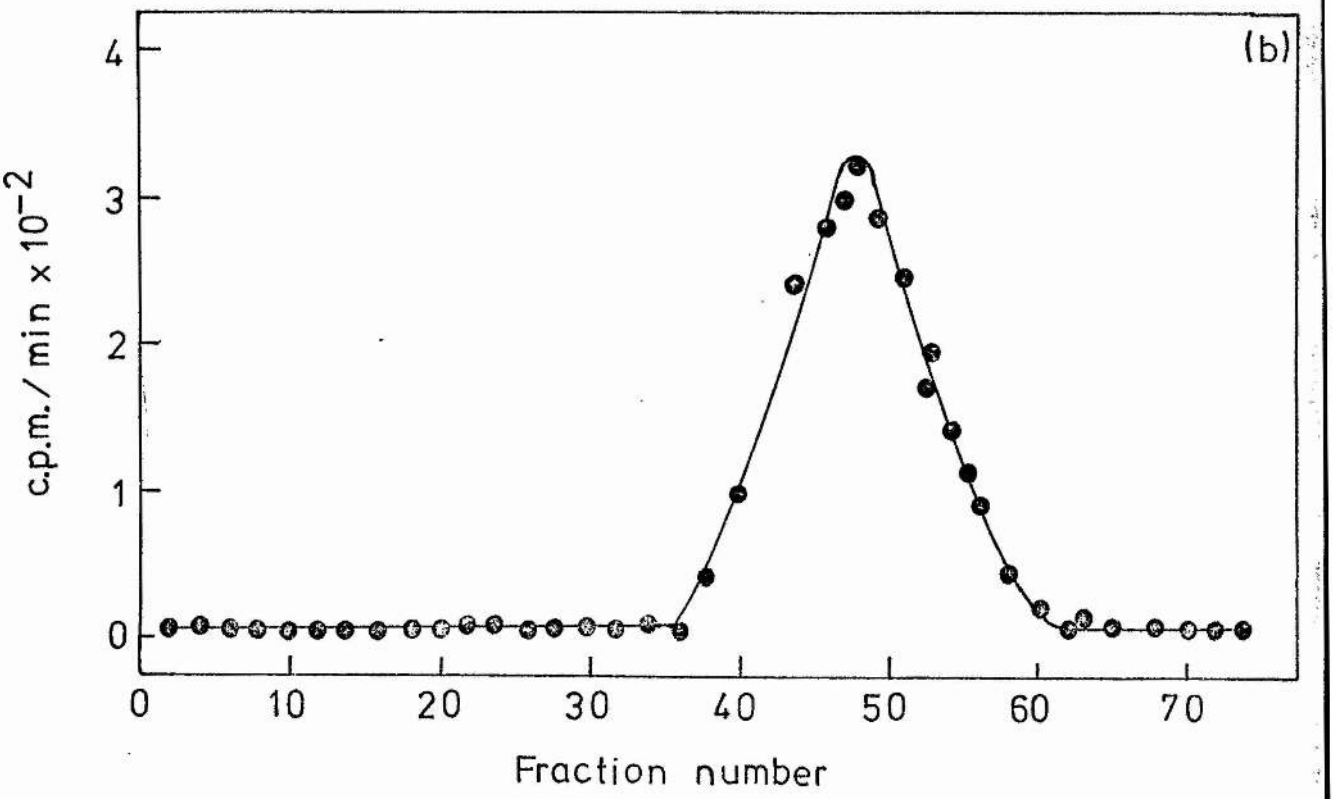
Demonstration of De Novo Synthesis and Turnover of Leucine: tRNA Ligase.

(a) Development of ligase activity.

Approximately 3.6g of stationary phase cells which had been labelled by growth for at least 100 generations in M-1D medium containing ^{15}N-nitrate and 30% deuterium oxide (heavy medium) were transfered into 250 ml of the same medium for 48h to allow sufficient detectable enzyme activity to develop. Cells were then transfered to 600 ml of M-1D medium containing 100% H_2O and 2.5 mM

Fig. 28 Distribution of leucine: tRNA ligase following isopycnic equilibrium centrifugation in caesium chloride, assayed either (a) by ATP-pyrophosphate exchange or (b) by aminoacylation of tRNA. Enzyme was extracted from 5 day old tobacco XD cells.


(a)
1·5
1·0
0·5
0
c.p.m. / min x 10^{-2}
0
10
20
30
40
50
60
70
Fraction number


(b)
4
3
2
1
0
c.p.m. / min x 10^{-2}
0
10
20
30
40
50
60
70
Fraction number

[^{14}N]-sodium nitrate as sole nitrogen source (light medium) and at this second transfer and subsequent time intervals, cells were harvested and enzyme activity determined (Fig. 29). An increase in the activity of leucine: tRNA ligase was obtained consistent with our previous observations (Fig. 14). Protein levels and specific activity are also shown.

(b) Evidence for de novo synthesis.

If the increase in activity of leucine: tRNA ligase is due to some type of enzyme activation then the buoyant density of the enzyme at all times after transfer into light medium should be identical with that of enzyme from fully labelled cells, since there would be no enzyme synthesis from (light) unlabelled amino acids synthesised in the light medium. Therefore at the same time intervals at which leucine: tRNA ligase activity was determined, the buoyant density of the enzyme was measured by isopycnic equilibrium centrifugation in caesium chloride. The distribution profiles that were obtained are shown in Fig. 30. They approximated to Gaussian distributions and therefore the density at the peak centre could be determined accurately by plotting the percentages representing enzyme activity on Gaussian graph paper (Onno, 1961). Such plots enable the peak centre to be defined by all the points on the enzyme distribution rather than those solely in the immediate vicinity of the peak region. Typical Gaussian plots of some of the data presented in Fig. 30 are shown in Fig. 31. The point of intersection at 100% corresponds to the fraction number at the peak centre. The refractive index, and therefore the buoyant density, can be determined by reference to the caesium chloride gradient formed during centrifugation. A typical example of such a caesium chloride gradient is shown in Fig. 32.

An examination of the buoyant densities determined from two experiments (Table 7) indicates that even at 6h after transfer

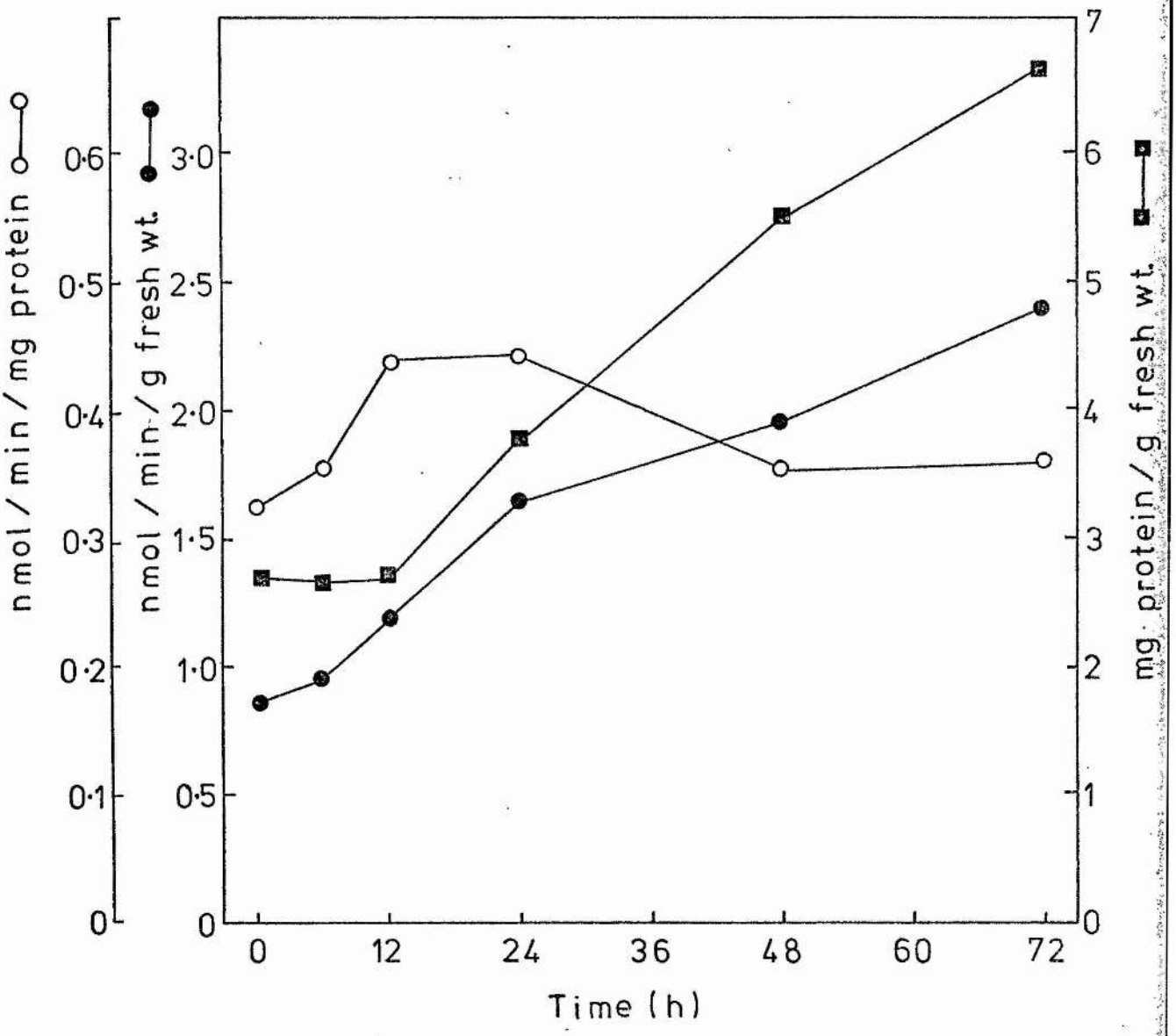


Fig. 29 Change in leucine: tRNA ligase activity ●——●, protein level ■——■ and specific activity O——O following transfer of 2 day old cells from heavy (labelled) M-1D to light (unlabelled) M-1D.

Fig. 30 The equilibrium distribution after centrifugation in caesium chloride of fully labelled (FL) and unlabelled (UL) leucine: tRNA ligase, and enzyme 6h, 12h, 48h, and 72h following transfer of 2 day old cells from heavy (labelled) M-1D into light (unlabelled) M-1D. Relative activity in this Fig. and where referred to in other Figs. means that all points on these curves are expressed as a percentage of the highest point on each of the individual curves.

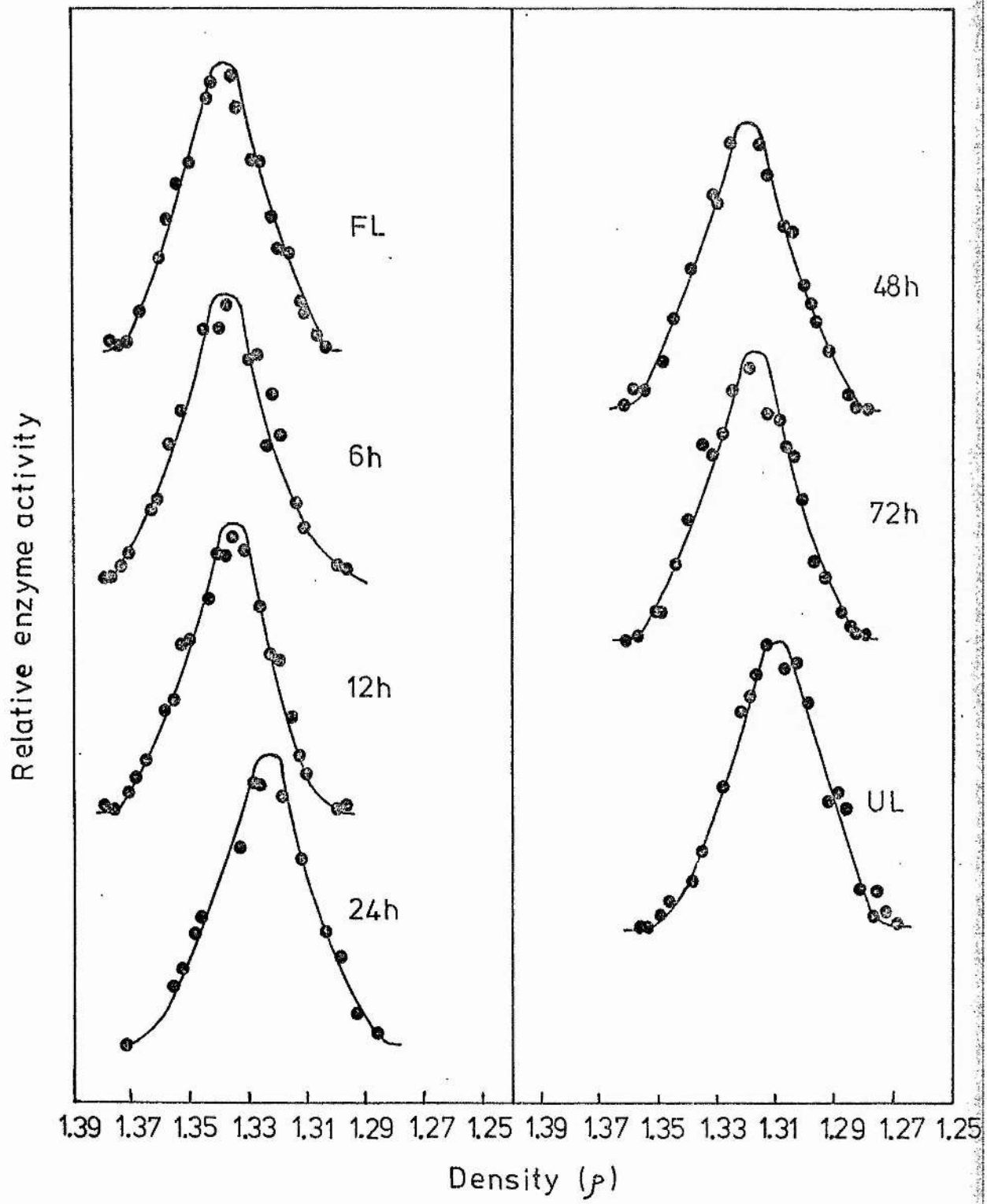

FL
6h
12h
24h
48h
72h
UL
Relative enzyme activity
1.39 1.37 1.35 1.33 1.31 1.29 1.27 1.25 1.39 1.37 1.35 1.33 1.31 1.29 1.27 1.25
Density (ρ)

Fig. 31 Gaussian plot of (a) fully labelled leucine: tRNA ligase and (b) leucine: tRNA ligase 72h in light (unlabelled) M-1D after transfer of 2 day old cells from heavy (labelled) M-1D.

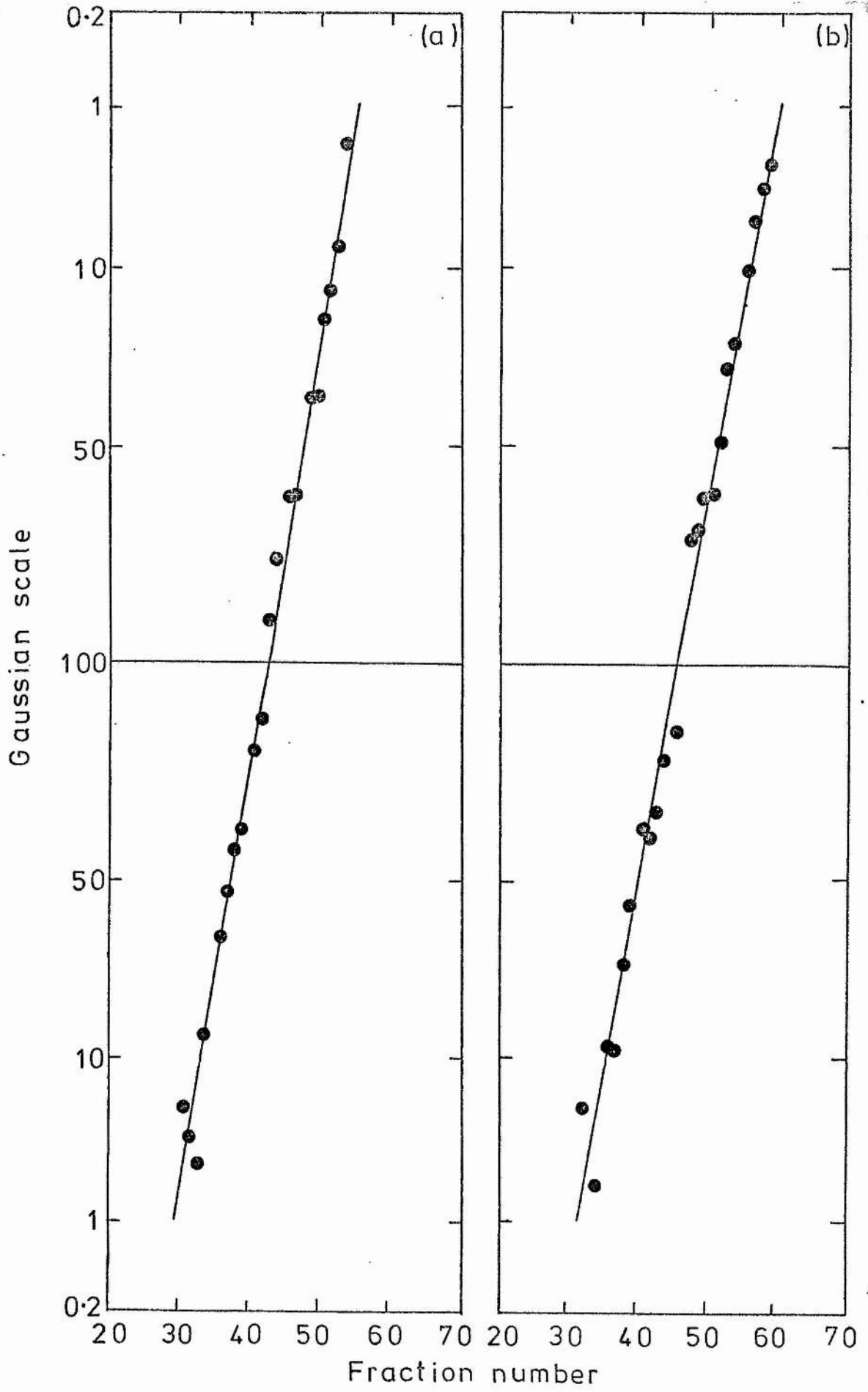

(a)
(b)
0·2
1
10
50
100
50
10
1
0·2
Gaussian scale
20
30
40
50
60
70
Fraction number

Fig. 32 A typical caesium chloride gradient formed after isopycnic equilibrium centrifugation of gradient mixture at 39,000 rpm (110,000g) for 65h.

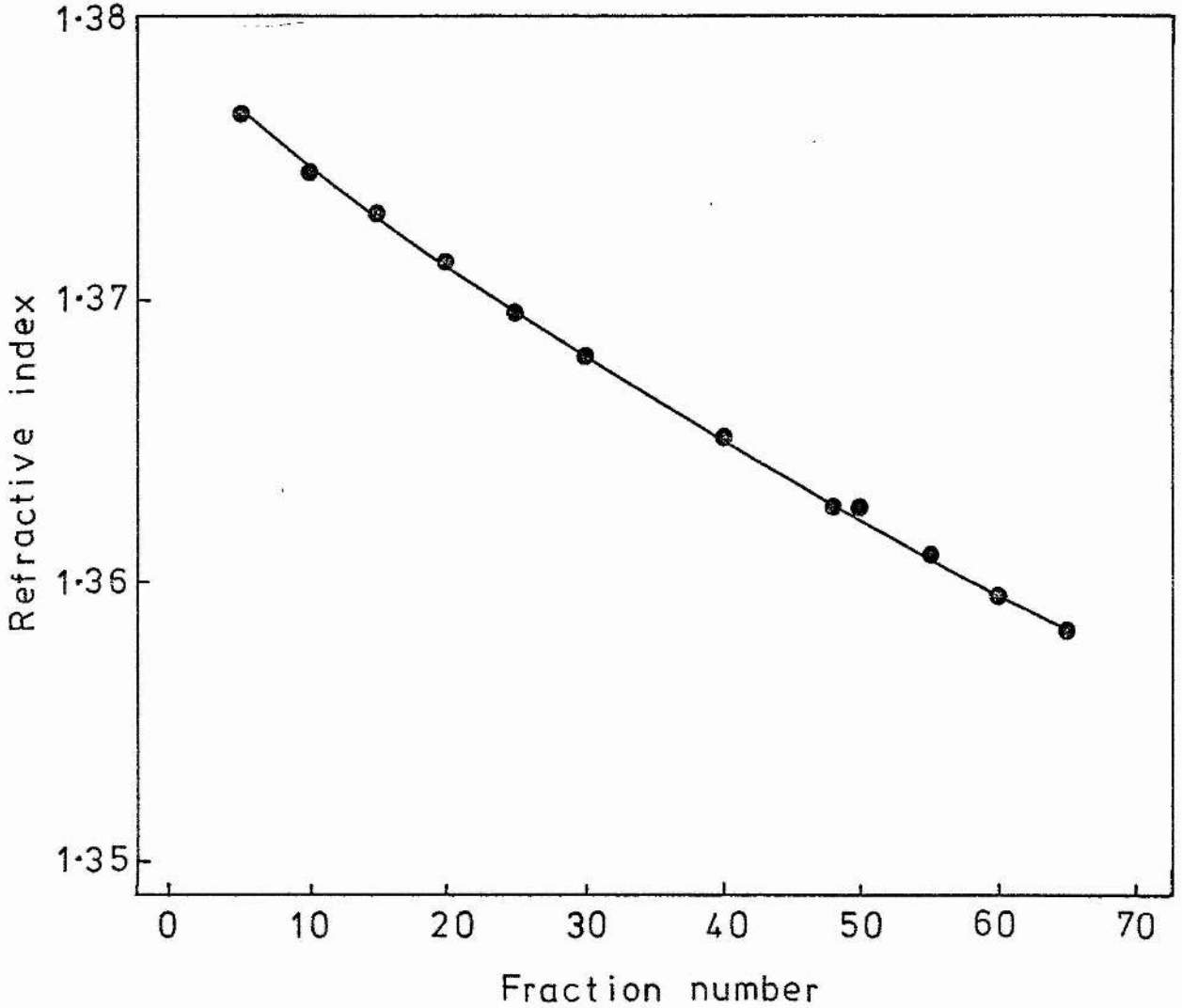

1·38
1·37
1·36
1·35
Refractive index
0
10
20
30
40
50
60
70
Fraction number

Table 7. Buoyant density and band width at half peak height of leucine: tRNA ligase following transfer to light M-1D medium.

Time after transfer into light medium	Band width at half peak height (no. of drops)			Buoyant density (ρ)		
	I	II	Average	I	II	Average
0 (Fully labelled)	31.2	33.0	32.1	1.3397	1.3397	1.3397
6	34.8	37.5	36.1	1.3364	1.3375	1.3370
12	35.4	33.3	34.3	1.3353	1.3331	1.3342
24	-	33.9	33.9	-	1.3234	1.3234
48	35.7	33.3	34.5	1.3201	1.3201	1.3201
72	34.5	34.5	34.5	1.3190	1.3158	1.3174
Unlabelled	35.7	32.4	34.0	1.3093	1.3103	1.3098

% increase of fully labelled enzyme over unlabelled enzyme = 2.3%

into light medium the buoyant density of leucine: tRNA ligase was less than the buoyant density of fully labelled enzyme. Therefore during the period that leucine: tRNA ligase increased in activity (Fig. 29) it was also synthesised de novo.

The observation that after transfer to light medium, the buoyant density of leucine: tRNA ligase decreased with time, and was intermediate between fully labelled and unlabelled enzyme, suggests that no synthesis occurred exclusively from light amino acids. Synthesis at early times after transfer was therefore largely from (heavy) labelled amino acids, derived either from a pre-existing ^{15}N-, ^{2}H- labelled amino acid pool or from degradation of pre-existing labelled protein and the re-incorporation of the resultant ^{15}N-, ^{2}H- labelled amino acids, or both (Zeilke & Filner, 1971). However at later time intervals synthesis occurred more and more from light amino acids, as these were synthesised. In other words synthesis of leucine: tRNA ligase occurred from an amino acid pool which became progressively richer in light amino acids.

The use of deuterium oxide as one of the density labels raises the possibility that were leucine: tRNA ligase a glycoprotein, the density shift observed after transfer to light medium could be entirely due to synthesis of a carbohydrate moiety rather than synthesis of the protein moiety. However, this is unlikely due to the low inherent density of the unlabelled enzyme (1.3098). For example horse radish peroxidase, an enzyme known to contain 20% carbohydrate has a buoyant density of 1.3490 (Quail & Scandalios, 1971). In addition the increase in density of fully labelled enzyme over unlabelled is 2.3% (Table 7), which agrees very well with the increase of 2.5% calculated for incorporation of label into enzyme protein.

c. Evidence for turnover.

The fact that the buoyant density of leucine: tRNA ligase falls with time after transfer to light medium is not evidence that the enzyme is also being degraded (i.e. turning over). For example, the position of the enzyme profile is governed by the relative proportions of unlabelled and labelled molecules in the enzyme population at any one time. Therefore if there was a rapid rate of synthesis in the post transfer period which elevated the proportion of new unlabelled molecules to a level several fold that of pre-existing labelled molecules, the buoyant density would decrease with time at a rate largely dependent on synthesis and not degradation.

However, if there were no degradation of pre-existing labelled enzyme molecules, a steadily increasing band width of the distribution profiles would be expected as more and more newly synthesised unlabelled molecules were added to the pre-existing labelled enzyme molecules (Quail & Scandalios, 1971). To resolve this problem the band widths at half peak height were determined from the Gaussian plots of the equilibrium centrifugation profiles, by extrapolating the 50% activity values to the ordinate axis (Fig. 30). At all times after transfer to light medium the band width remained essentially constant (Table 7), indicating that pre-existing heavy labelled leucine: tRNA ligase molecules were degraded during the period of enzyme synthesis and that therefore leucine: tRNA ligase turns over. Presumably at later time intervals after transfer lighter enzyme molecules will also be degraded.

In the most extreme case where little protein turnover occurs i.e. in exponentially growing bacterial cells (Willetts, 1967), the development of a discrete separate peak of unlabelled enzyme can be followed after transfer of cells labelled by growth in 80% deuterium oxide into light medium (McGinnis & Williams, 1971).

Evidence for De Novo Synthesis and Turnover of Arginine: tRNA Ligase.

To determine whether de novo synthesis and turnover of leucine: tRNA ligase was unique amongst the amino acid: tRNA ligases in XD cells, similar experiments to those previously described for the leucine enzyme were performed for arginine: tRNA ligase.

(a) Development of ligase activity and evidence for de novo synthesis.

Stationary phase cells, labelled by growth in heavy medium as previously described, were transfered into the same medium for 48h and then transfered into light medium. At this second transfer and subsequent time intervals, cells were harvested and enzyme activity determined (Fig. 33). As expected there was an increase in arginine: tRNA ligase activity consistent with our previous observations (Fig. 14). At these same time intervals enzyme was subjected to isopycnic equilibrium centrifugation to determine if during this period of activity increase arginine: tRNA ligase was synthesised de novo. The distribution profiles so obtained are shown in Fig. 34 and again approximated to Gaussian distributions. Gaussian plots of these profiles were therefore used to accurately determine the buoyant density of the enzyme after transfer to light medium. Typical Gaussian plots of two of the distribution profiles are shown in Fig. 35. The buoyant densities obtained in two experiments are presented in Table 8.

After 12h in light medium the buoyant density of arginine: tRNA ligase had decreased (Table 8), indicating that like the leucine enzyme it was synthesised de novo. The buoyant density again decreased with time and was intermediate between fully labelled and unlabelled enzyme, suggesting that synthesis was not occurring

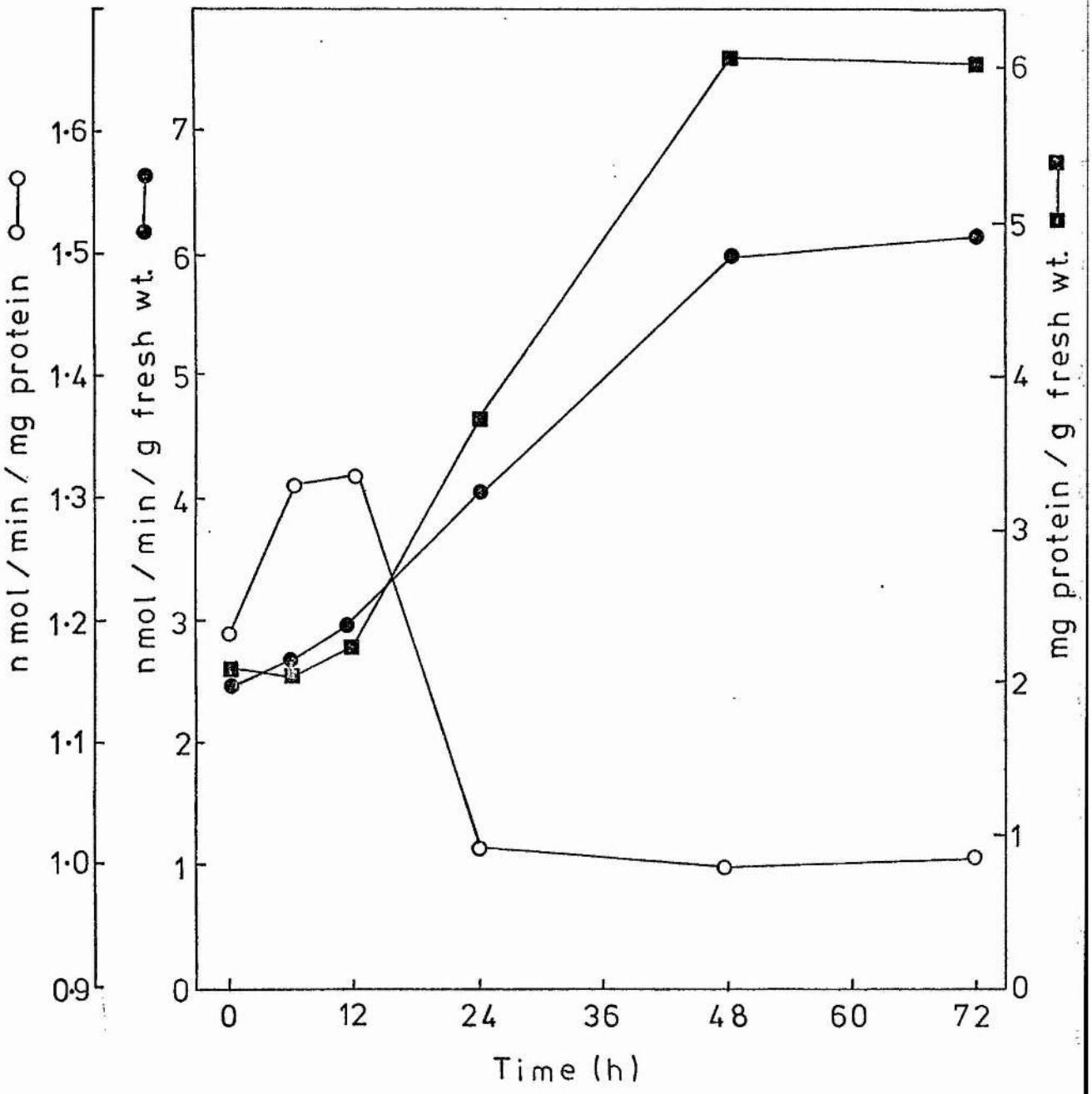


Fig. 33 Change in arginine: tRNA ligase activity ●——●, protein level ■——■ and specific activity ○——○ following transfer of 2 day old cells from heavy (labelled) M-1D to light (unlabelled) M-1D.

Fig. 34 The equilibrium distribution after centrifugation in caesium chloride of fully labelled (FL) and unlabelled (UL) arginine: tRNA ligase, and enzyme 6h, 12h, 48h and 72h following transfer of 2 day old cells from heavy (labelled) M-1D into light (unlabelled) M-1D.

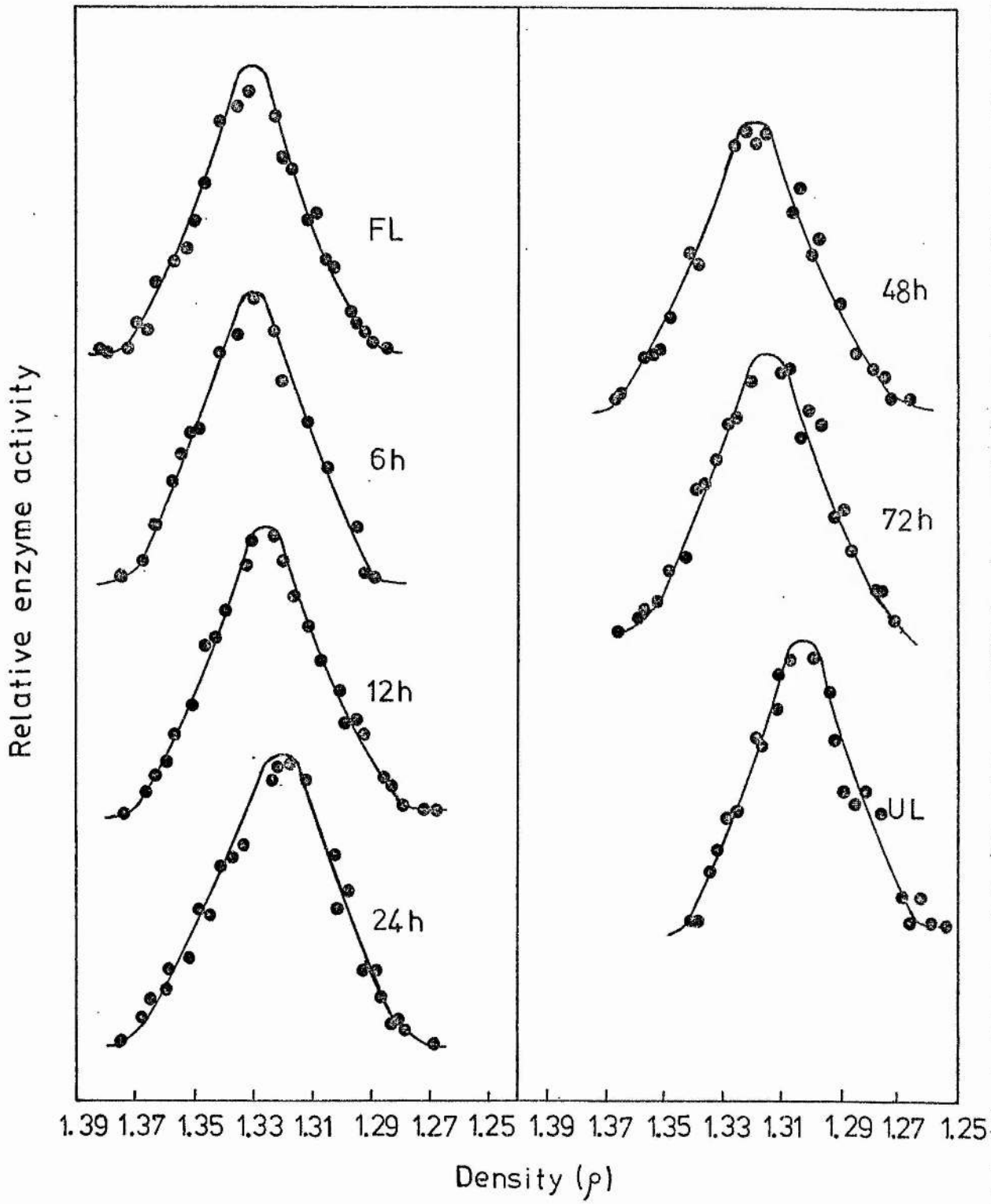

FL
6h
12h
24h
48h
72h
UL
Relative enzyme activity
1.39 1.37 1.35 1.33 1.31 1.29 1.27 1.25
1.39 1.37 1.35 1.33 1.31 1.29 1.27 1.25
Density (ρ)

Fig. 35 Gaussian plot of (a) fully labelled arginine: tRNA ligase and (b) arginine: tRNA ligase 72h in light (unlabelled) M-1D after transfer of 2 day old cells from heavy (labelled) M-1D into light (unlabelled) M-1D.

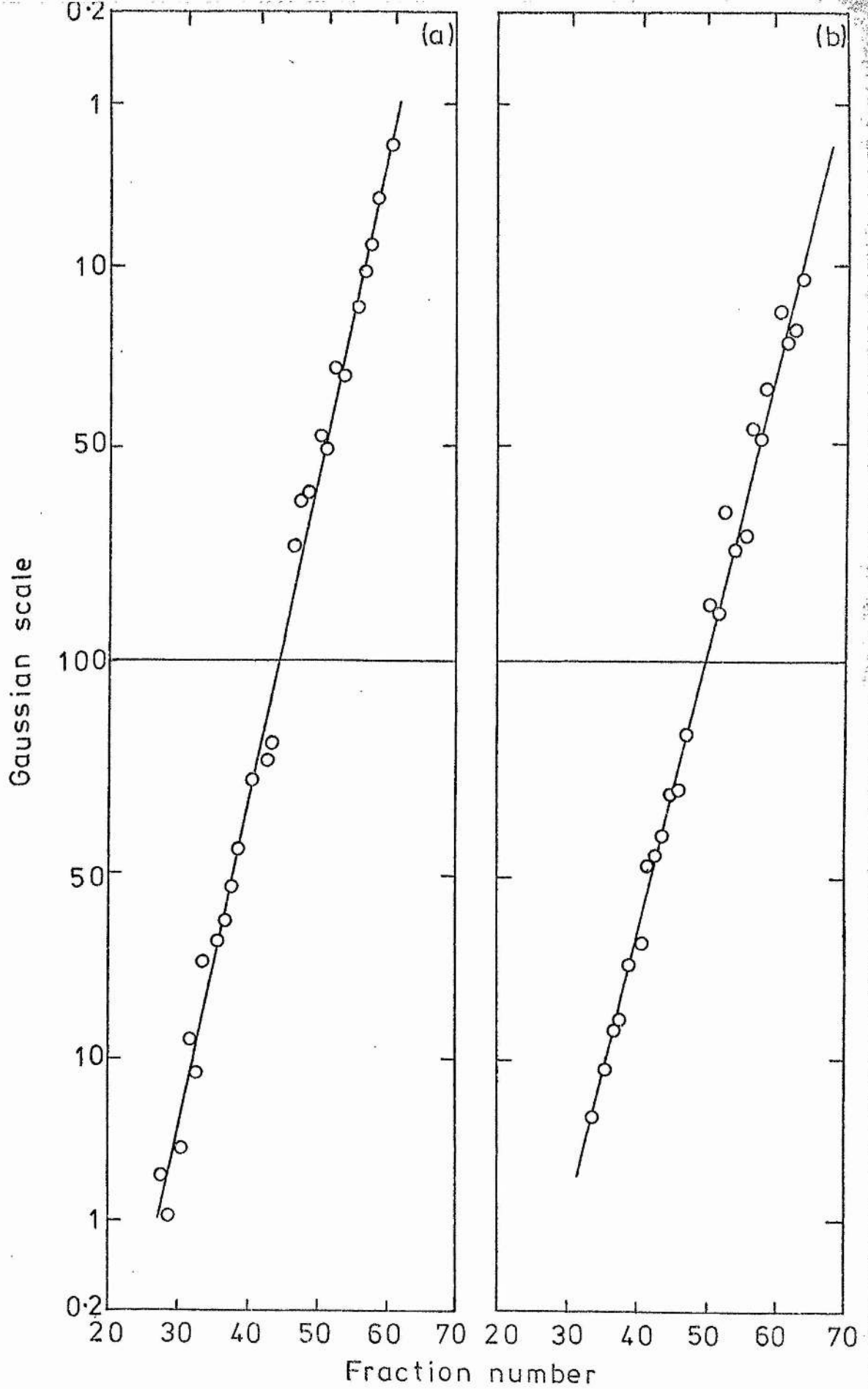

(a)
(b)
0·2
1
10
50
100
50
10
1
0·2
Gaussian scale
20
30
40
50
60
70
Fraction number

Table 8. Buoyant density and band width at half peak height of arginine: tRNA ligase following transfer to light M-1D medium.

	Time after transfer to light medium	Band width at half peak height (no. of drops)	Buoyant density (ρ)
(a)	0 (fully labelled)	40.2	1.3288
	6	43.2	1.3288
	12	42.6	1.3234
	24	48.9	1.3201
	48	45.9	1.3190
	72	45.3	1.3136
	Unlabelled	36.0	1.3055

% increase of fully labelled enzyme over unlabelled enzyme = 1.8%

(b)	0 (fully labelled)	42.9	1.3240
	6	47.1	1.3240
	12	48.6	1.3185
	24	54.0	1.3158
	48	54.0	1.3147
	72	45.3	1.3114
	Unlabelled	36.0	1.3055

exclusively from light amino acids. However, between 0 and 6h after transfer (Table 8) no decrease in buoyant density was observed and therefore enzyme would appear to be synthesised wholly from labelled amino acids. This implies that newly synthesised light amino acids do not equilibrate with the amino acid precursor pool for this enzyme until after 6h in light medium. However, the buoyant density of leucine: tRNA ligase decreased during this period (Table 7), suggesting that the two enzymes may be synthesised from different amino acid pools.

Although a buoyant density of 1.3288 was obtained for fully labelled enzyme on three separate occasions, in the second density labelling experiment (Table 8b), fully labelled enzyme had a buoyant density of only 1.3240. This difference is difficult to explain, since prior to both experiments the cells had been grown in heavy medium for at least 100 generations, and in addition, the cells used for the second experiment had been in heavy medium for a longer period than those used for the first. However, as will be seen in the general discussion, the rate at which the density shifts to the unlabelled state is similar in both cases.

The increase in density of fully labelled arginine: tRNA ligase over unlabelled is 1.8% compared with 2.3% found for the leucine enzyme. The two enzymes, therefore, might have different amino acid compositions. Leucine: tRNA ligase molecules, for example, might be synthesised from more amino acids such as arginine, histidine, lysine and tryptophan which have an additional ^{14}N replaceable by ^{15}N.

(b) Evidence for tunrover.

As already discussed for leucine: tRNA ligase a change in the buoyant density with time after transfer to light medium is not

evidence for degradation of pre-existing labelled enzyme molecules. However, a constant band width at all times after transfer, similar to that found for leucine: tRNA ligase would conclusively demonstrate that the enzyme was being degraded and therefore turning over. The band widths at half peak height were determined as previously described from Gaussian plots of the equilibrium centrifugation profiles and are presented in Table 8. The band widths are broader than those found for the leucine enzyme and are not constant. There is for example a 14% band broadening at 24h. The data does not therefore allow the conclusion that arginine: tRNA ligase is continually turning over. However, if there were no degradation of labelled enzyme molecules then a steadily increasing band width might be expected as more and more unlabelled enzyme molecules were added to pre-existing labelled molecules. This is also not found. It could be argued therefore that either arginine: tRNA ligase does not turn over continuously or at 24h something else is happening to account for the band broadening. It seems unlikely that degradation and therefore turnover of enzyme molecules will stop and then restart a few hours later. Another explanation is that for some reason at 24h there is a substantial increase in the population of lighter labelled enzyme molecules.

We have already discussed in the general introduction the existence in plant tissues of multiple amino acid: tRNA ligases cognate for a particular amino acid. The occurrence of such multiple species could explain the band width data in terms of organelle specific species and/or multiple species turning over at different rates. The existence of such species is investigated in the next section.

In conclusion, whereas it is clear that leucine: tRNA ligase turns over, the situation for the arginine enzyme appears

to be more complex and will be discussed more fully in the general discussion.

Evidence for De Novo Synthesis and Turnover of Leucine: and Arginine: tRNA Ligases Under Conditions of Nitrate Restriction.

The density labelling technique was also used to examine whether leucine: and arginine: tRNA ligases are synthesised de novo and turn over under conditions of nitrate restriction.

(a) Evidence for de novo synthesis.

Approximately 4g of stationary phase cells, labelled in the normal way, were subcultured into 1 litre of light nitrateless medium. Immediately after transfer and at subsequent time intervals cell samples were taken, and measurements of activity and of buoyant density by isopycnic equilibrium centrifugation were performed for the two enzymes. The activities of both enzymes remained fairly constant after transfer (Fig. 36). As might be expected the distribution profiles obtained with leucine: tRNA ligase (Fig. 37) and arginine: tRNA ligase (Fig. 38) approximated to Gaussian distributions. Gaussian plots of some of the distribution profiles are shown in Fig. 39 and Fig. 40. The buoyant densities obtained from such plots are presented in Table 9 and the results from two experiments are included for leucine: tRNA ligase (Table 9a). The buoyant densities of both enzymes fell with time, indicating that as in M-1D medium, they were both synthesised de novo and not synthesised exclusively from light amino acids.

We again find that the buoyant density of arginine: tRNA ligase does not decrease immediately after transfer to light medium (Table 9b). This tends to confirm our previous hypothesis that leucine: tRNA ligase is synthesised from a different amino acid pool, since under two different growth conditions i.e. when 2 day old cells are transfered to light M-1D medium and stationary phase

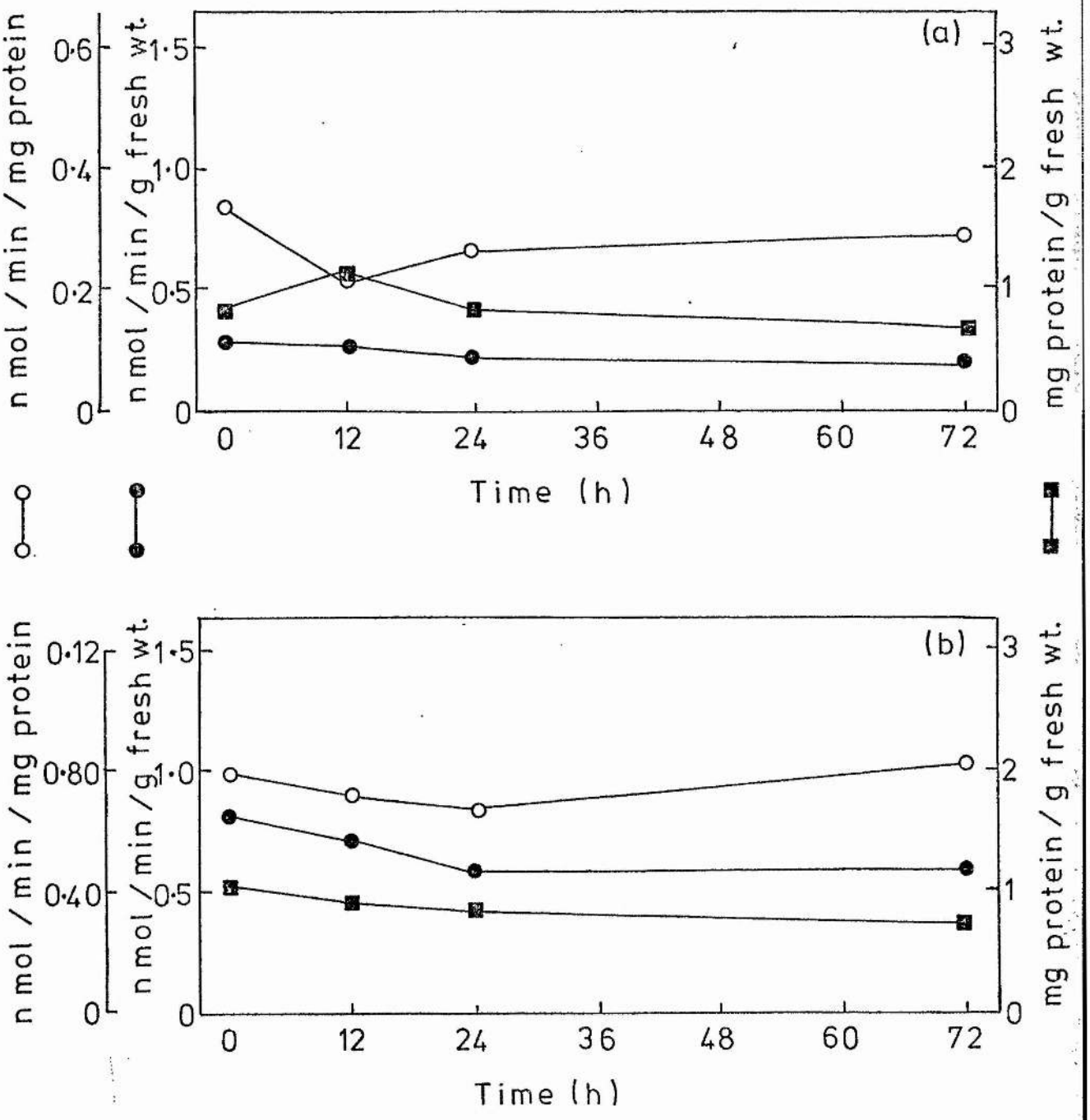


Fig. 36 Change in (a) leucine: tRNA ligase activity and (b) arginine: tRNA ligase activity ●—● protein level ■—■ and specific activity ○—○ following transfer of stationary cells from heavy (labelled) M-1D into light (unlabelled) nitrateless M-1D.

Fig. 37 The equilibrium distribution after centrifugation in caesium chloride of fully labelled (FL) and unlabelled (UL) leucine: tRNA ligase, and enzyme 12h, 24h and 72h following transfer of stationary phase cells from heavy (labelled) M-1D into light (unlabelled) nitrateless M-1D.

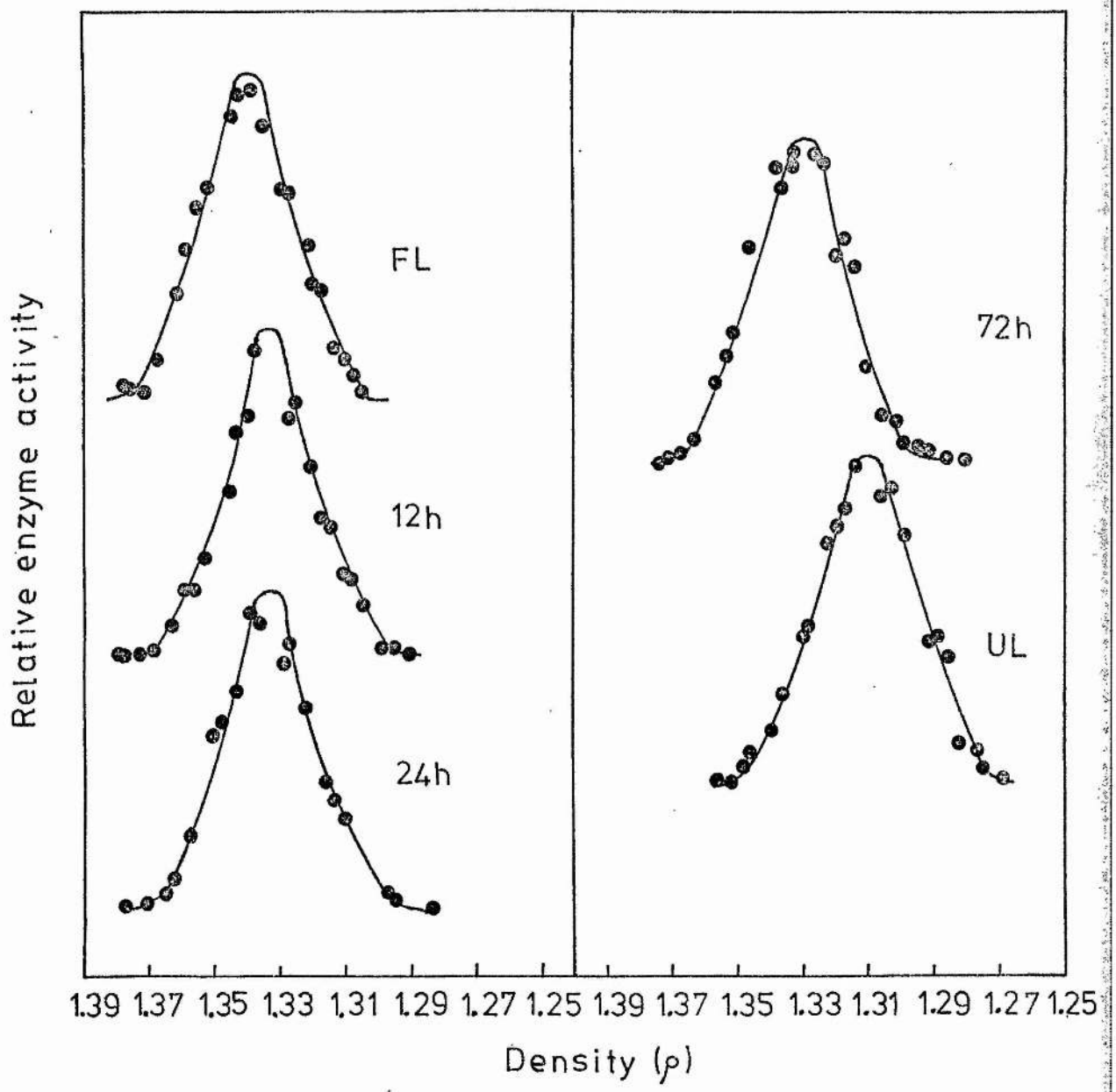

Relative enzyme activity
FL
12h
24h
72h
UL
1.39 1.37 1.35 1.33 1.31 1.29 1.27 1.25
1.39 1.37 1.35 1.33 1.31 1.29 1.27 1.25
Density (ρ)

Fig. 38 The equilibrium distribution after centrifugation in caesium chloride of fully labelled (FL) and unlabelled (UL) arginine: tRNA ligase, and enzyme 12h, 24h and 72h following transfer of stationary phase cells from heavy (labelled) M-1D into light (unlabelled) nitrateless M-1D.

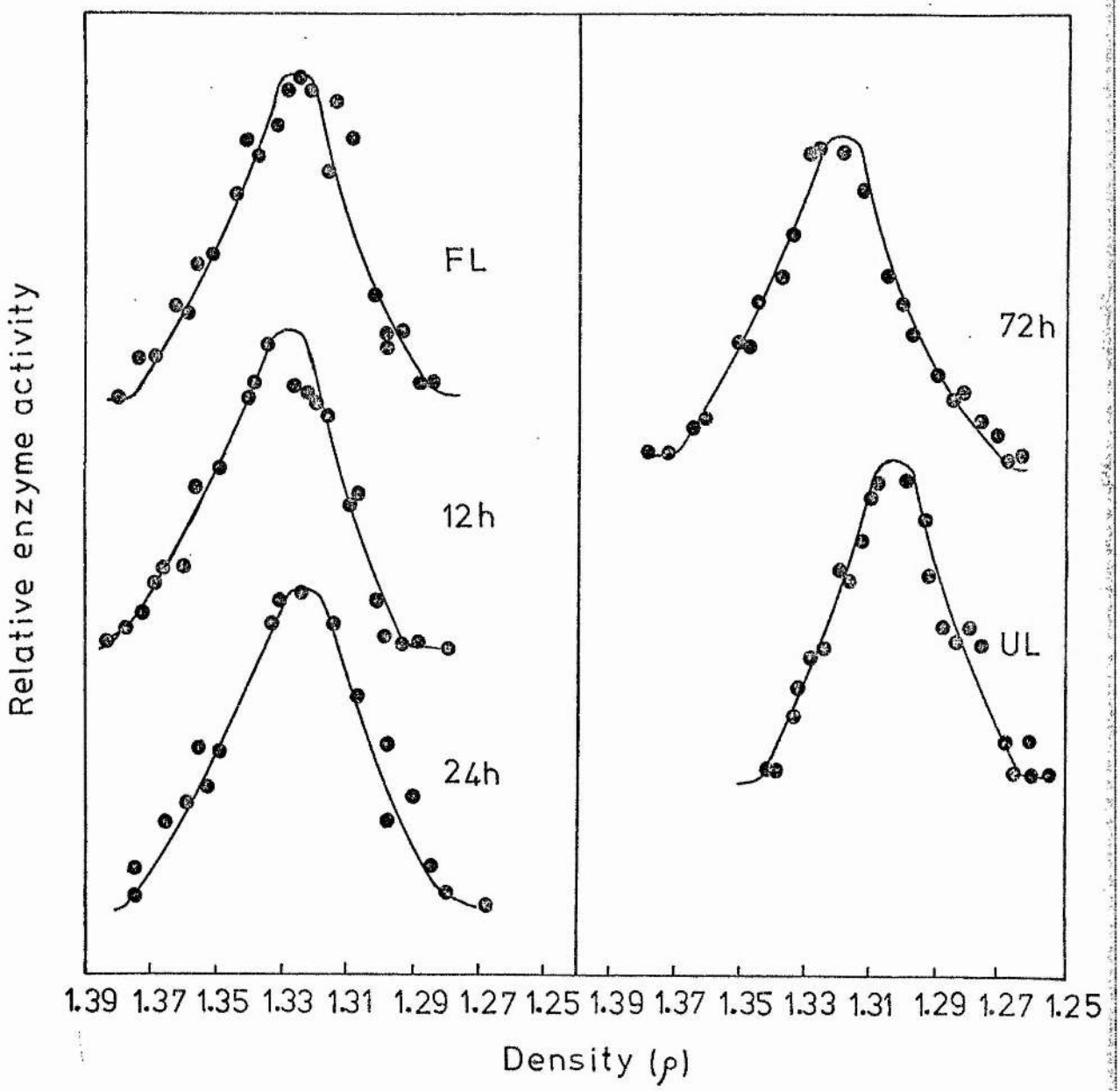

Relative enzyme activity
FL
12h
24h
72h
UL
1.39 1.37 1.35 1.33 1.31 1.29 1.27 1.25 1.39 1.37 1.35 1.33 1.31 1.29 1.27 1.25
Density (ρ)

Fig. 39 Gaussian plot of (a) fully labelled leucine: tRNA ligase and (b) leucine: tRNA ligase 72h in light (unlabelled) nitrateless M-1D after transfer of stationary phase cells from heavy (labelled) M-1D.

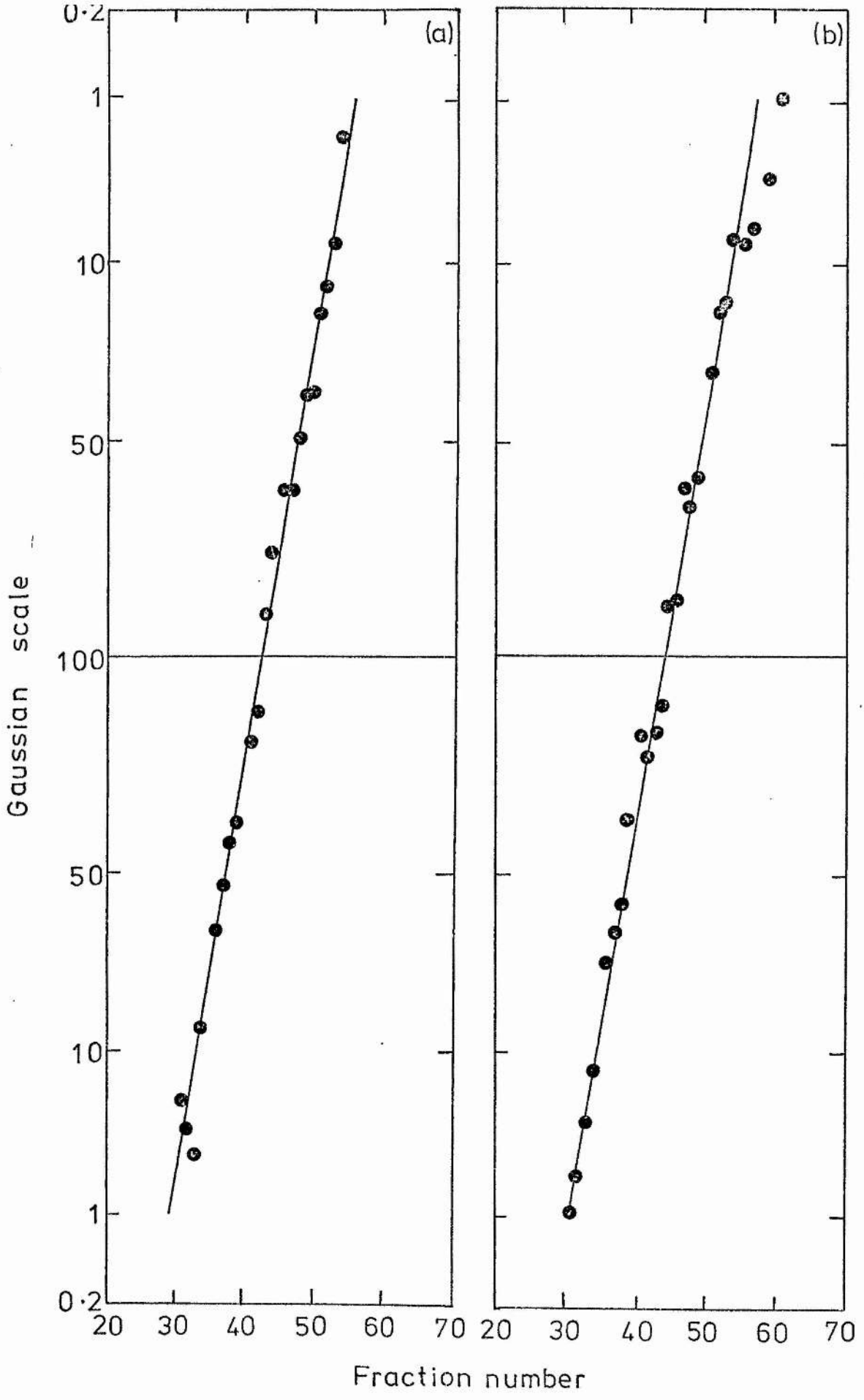

(a)
(b)
0·2
1
10
50
100
50
10
1
0·2
Gaussian scale
20
30
40
50
60
70
Fraction number

Fig. 40 Gaussian plot of (a) fully labelled arginine: tRNA ligase and (b) arginine: tRNA ligase 72h in light (unlabelled) nitrateless M-1D after transfer of stationary phase cells from heavy (labelled) M-1D.

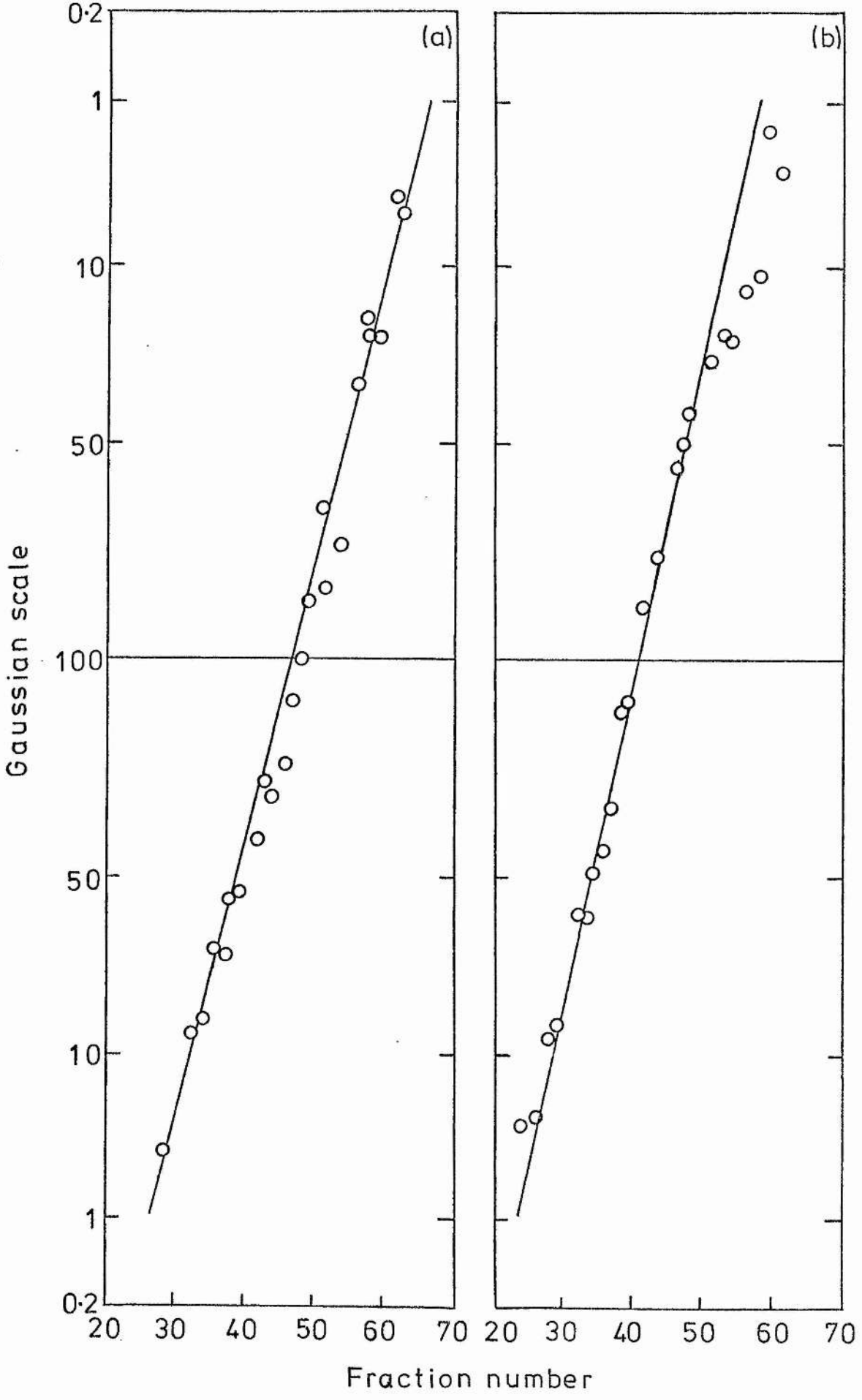

(a)
(b)
0·2
1
10
50
100
50
10
1
0·2
Gaussian scale
20
30
40
50
60
70
Fraction number

Table 9. Buoyant density and band width at half peak height of (a) leucine: tRNA ligase and (b) arginine: tRNA ligase after transfer to light nitrateless M-1D medium.

(a)

Time after transfer into light nitrateless M-1D medium	Band width at half peak height (no. of drops)			Buoyant density (ρ)		
	I	II	Average	I	II	Average
0 (Fully labelled)	31.2	33.0	32.1	1.3397	1.3397	1.3397
12	-	29.7	29.7	-	1.3342	1.3342
24	34.5	29.1	31.8	1.3299	1.3315	1.3308
72	30.0	31.5	30.8	1.3288	1.3293	1.3291
Unlabelled	35.7	32.4	34.0	1.3093	1.3103	1.3098

(b)

Time after transfer into light nitrateless M-1D medium	Band width at half peak height (no. of drops)	Buoyant density (ρ)
0 (Fully labelled)	46.5	1.3288
12	42.4	1.3288
24	41.4	1.3256
72	49.5	1.3201
Unlabelled	36.0	1.3055

cells are transferred to light nitrateless medium, no initial change in buoyant density of arginine: tRNA ligase was observed. A similar argument can be used against there being activation of pre-existing enzyme in this immediate post transfer period.

The decrease in density of both enzymes after 72h in light nitrateless M-1D medium (Table 9) is only about 50% of that found in light M-1D medium (Table 7 and Table 8). This is because when cells are transferred to light nitrateless M-1D medium all the nitrogen in the cells remains in the form of ^{15}N, as no light ^{14}N can be introduced after transfer. Therefore heavy amino acids can only become lighter by transamination and interconversion with light hydrogen and heavy nitrogen amino acids, synthesised from the sucrose in the medium.

(b) Evidence for turnover.

Since there was no increase in the activities of both arginine: and leucine: tRNA ligase after transfer to light nitrate-less medium (Fig. 36), yet they were both being synthesised de novo, degradation must also have been occurring and therefore both enzymes turn over. Evidence to support this conclusion should be obtained from the band widths determined from Gaussian plots of the equilibrium distribution profiles. The band widths of leucine: tRNA ligase (Table 9a) were essentially constant after transfer to light nitrateless medium, consistent with the enzyme turning over. A similar result should be expected for arginine: tRNA ligase. However, the band widths were again much more variable (Table 9b). This indicates that the band width data of arginine: tRNA ligase is not a very satisfactory parameter for providing evidence for turnover of this enzyme. It also confirms our earlier hypothesis that although the band widths were not constant when arginine: tRNA

ligase was transfered to light M-1D medium, this did not necessarily mean that the enzyme failed to turnover continuously. The existence of multiple ligase species cognate for arginine to account for these observations is investigated in the subsequent section.

SECTION 4

EVIDENCE FOR MULTIPLE FORMS OF ARGININE: AND LEUCINE: tRNA LIGASES

INTRODUCTION

In the general introduction, we discussed the observation that in a number of plant tissues multiple amino acid: tRNA ligases cognate for a particular amino acid had been reported and that this was not surprising, since both chloroplasts and mitochondria were able to synthesise part of their own protein complement.

In this section we have looked for the presence of such multiple species cognate for arginine and leucine in protein extracted from XD cells in lag phase, exponential phase or in nitrateless medium. It was hoped, therefore, that the density labelling data, particularly with respect to the band widths obtained with arginine: tRNA ligase, could be explained in terms of multiple enzymic species.

RESULTS AND DISCUSSION

The possibility that enzyme extracts of arginine: and leucine: tRNA ligases consisted of multiple species was examined by fractionating enzyme preparations on DEAE cellulose with a linear salt gradient.

Characterisation of the Gradient and Determination of Salt Concentration Required to Elute Protein from DEAE Cellulose

Our mixing vessels were examined for their ability to establish a linear gradient by observing the mixing of methylene blue with distilled water. The mixing chamber contained 120ml of distilled water and the reservoir contained 120ml of 30μM methylene blue. Approximately 80 3ml fractions were collected and the absorbance reading at 600nm of every 5th fraction was determined. The straight line obtained by plotting absorbance reading against fraction number (Fig. 41), indicated that these mixing vessels produced a linear gradient and were therefore suitable for our experiments.

In preliminary DEAE cellulose chromatography experiments, it was found that if mercaptoacetate was used to maintain reducing conditions, the rapid oxidation of the sulphydryl group interfered with absorbance readings at 280nm and prevented protein from being monitored. The use of mercaptoethanol, however, was found to be satisfactory.

The concentration of salt required to elute protein from a DEAE cellulose column was determined by extracting 80mg of protein as described in the materials and methods section. The protein was added to the column and washed excessively with 70ml of potassium phosphate buffer, pH 7.5 containing 10mM mercaptoethanol followed by 50ml of the same buffer containing 0.2M KCl and 70ml containing 0.4M KCl. 7ml fractions were

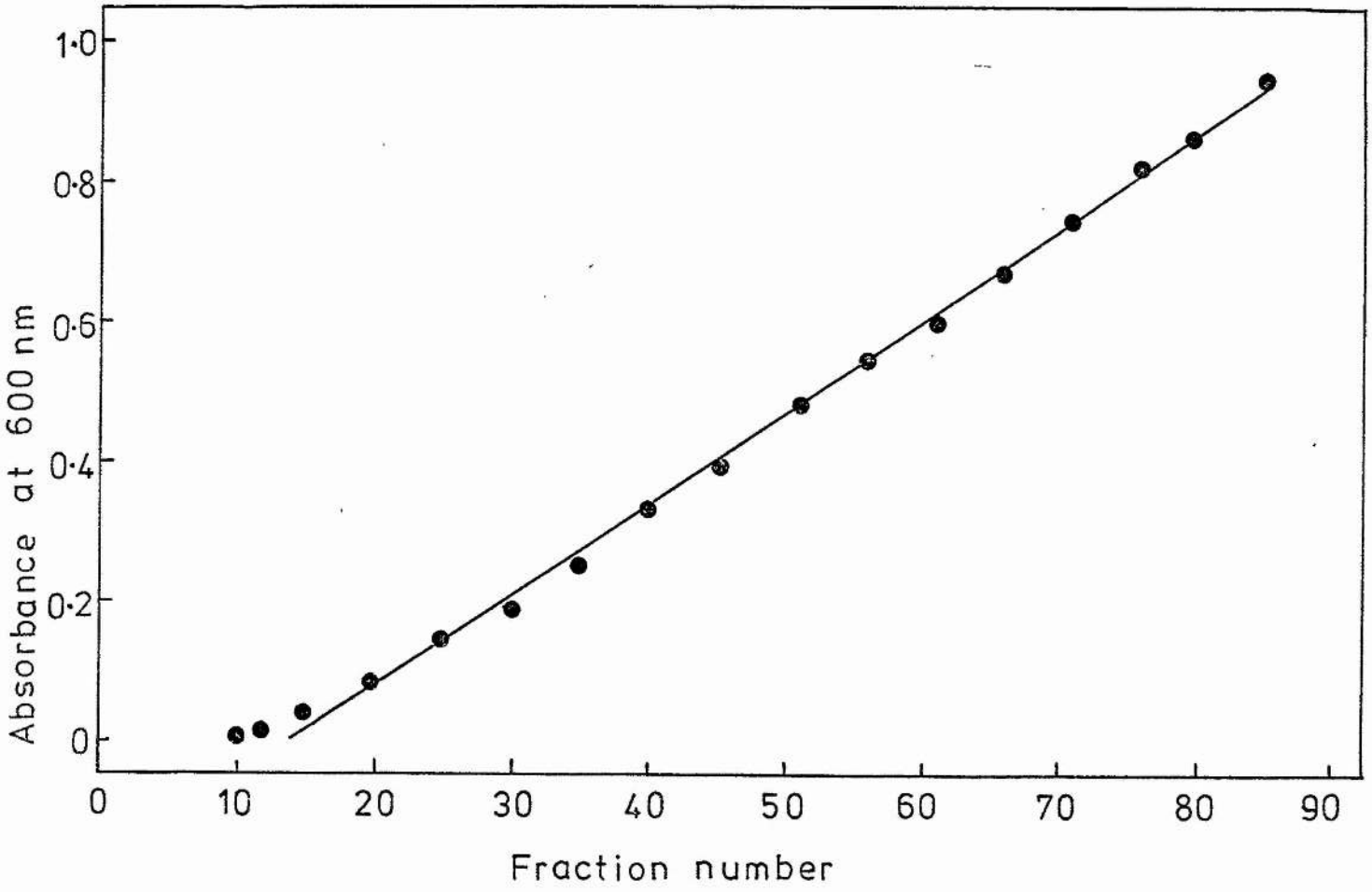


Fig. 41 Establishment of a linear gradient when methylene blue is mixed with distilled water.

collected and the absorbance readings at 280nm monitored (Fig. 42). Some protein did not adsorb to the column but was washed through with the buffer. The majority, however, adsorbed to DEAE cellulose and was eluted by 0.2M KCl, although a small amount appeared to be removed by 0.4M KCl. In most subsequent experiments, therefore, a 0-0.3M linear KCl gradient was used to elute protein from DEAE cellulose.

DEAE Cellulose Chromatography of Enzyme Extracted from Cells Grown for 7 Days in M-1D (i.e. Cells in Exponential Phase)

There are many reports of multiple iso-accepting tRNA species for an amino acid, some of which are organelle specific (Burkard et al., 1970; Guderian et al., 1972; Guillemaut et al., 1975) and some of which may change during growth and development of plant tissues (Vold & Sypherd, 1968; Bick et al., 1970). In some cases, multiple enzymes for a single amino acid may have different activities towards different iso-accepting tRNA species (Barnett & Epler, 1966; Burkard et al., 1970; Guillemaut et al., 1975). In this thesis we have used 7 day tRNA to assay amino acid: tRNA ligases. Therefore, in view of the above observations, it was felt that detection of multiple enzymic species should initially be carried out under homologous assay conditions i.e. where tRNA and enzyme are extracted from cells of the same age. This would ensure detection of all ligase species present in the protein extract.

Approximately 80mg of protein was therefore extracted from XD cells grown for 7 days in M-1D medium, as described in the materials and methods section, and adsorbed onto DEAE cellulose. The column was washed with 50-60ml of potassium phosphate buffer, pH 7.5 containing 10mM mercaptoethanol, prior to protein elution with a 0-0.3M KCl gradient. Approximately 80 3ml fractions were

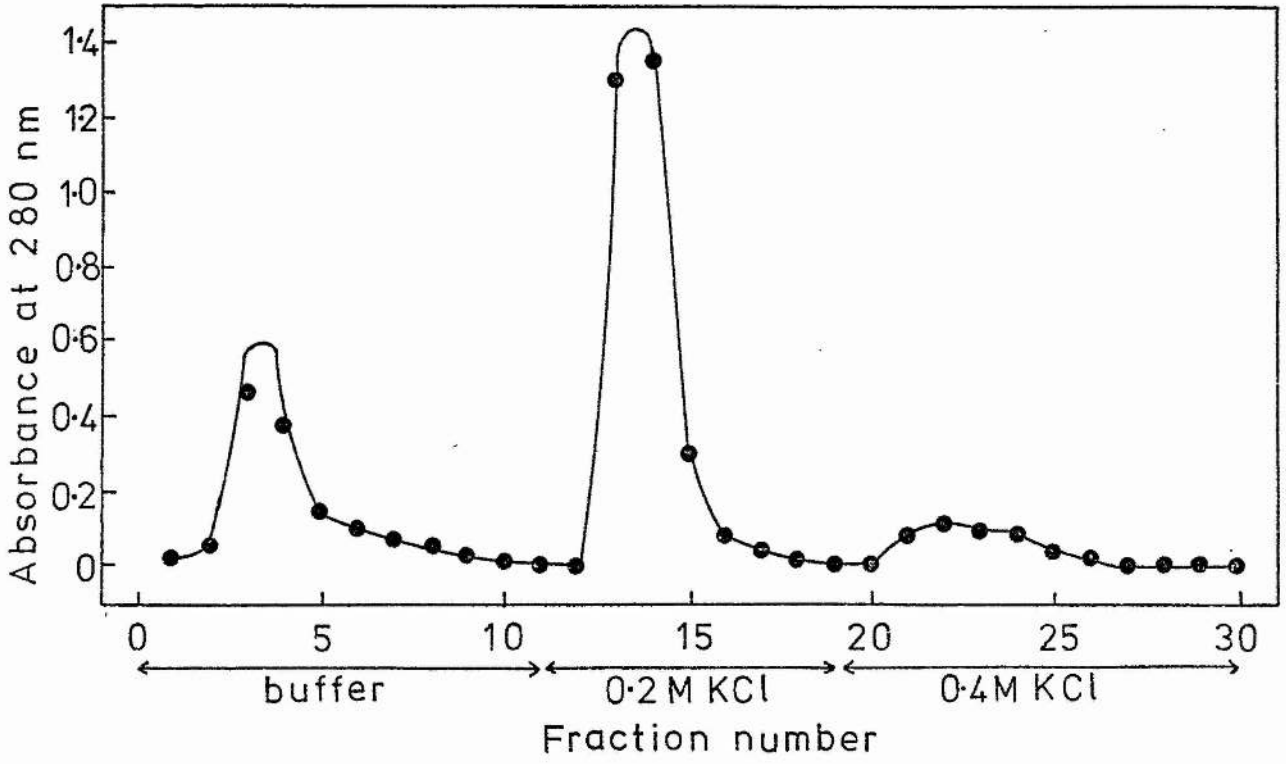


Fig. 42 Elution of protein from DEAE cellulose by 0.2M KCl and 0.4M KCl

collected after the application of the gradient and the distribution of arginine: and leucine: tRNA ligases determined from the assay of fractions by aminoacylation of tRNA.

Arginine: tRNA ligase appeared to fractionate into three activity regions, I, II and III (Fig. 43) whereas the distribution of the leucine enzyme (Fig. 44) consisted of one major activity region, B and another much smaller region, A.

Since the arginine: tRNA ligase regions I and II were poorly separated, elution from DEAE cellulose with a shallower KCl gradient would be expected to result in a better separation of the two activity regions. Therefore the tubes containing these two activity regions (fraction numbers 32-49) were pooled and the protein precipated by adjusting to 75% saturation with a saturated solution of ammonium sulphate. After dialysis for 15h to remove any remaining ammonium sulphate, the protein was re-chromatographed on DEAE cellulose with a 0.03-0.18M KCl gradient. As expected, there was a better separation of these two activity regions (Fig. 45), confirming that they were separate and distinct species.

A similar experiment was performed to improve the separation of the two leucine: tRNA ligase species. Tubes containing the two activity regions (fraction numbers 50-65) were pooled and after protein precipitation with ammonium sulphate and subsequent dialysis, the protein was re-chromatographed on DEAE cellulose with a 0.1-0.25M KCl gradient. The distribution of enzyme activity clearly showed that there were two distinct activity regions (Fig. 46).

It would appear, therefore, that arginine: tRNA ligase extracted from 7 day old cells is composed of three species of approximately equal proportions, whereas leucine: tRNA ligase is

Fig. 43 Distribution of arginine: tRNA ligase activity ●——● and protein O——O following DEAE cellulose fractionation of protein extracted from cells grown for 7 days in M-1D. Protein was eluted from DEAE cellulose with a 0-0.3M KCl gradient.

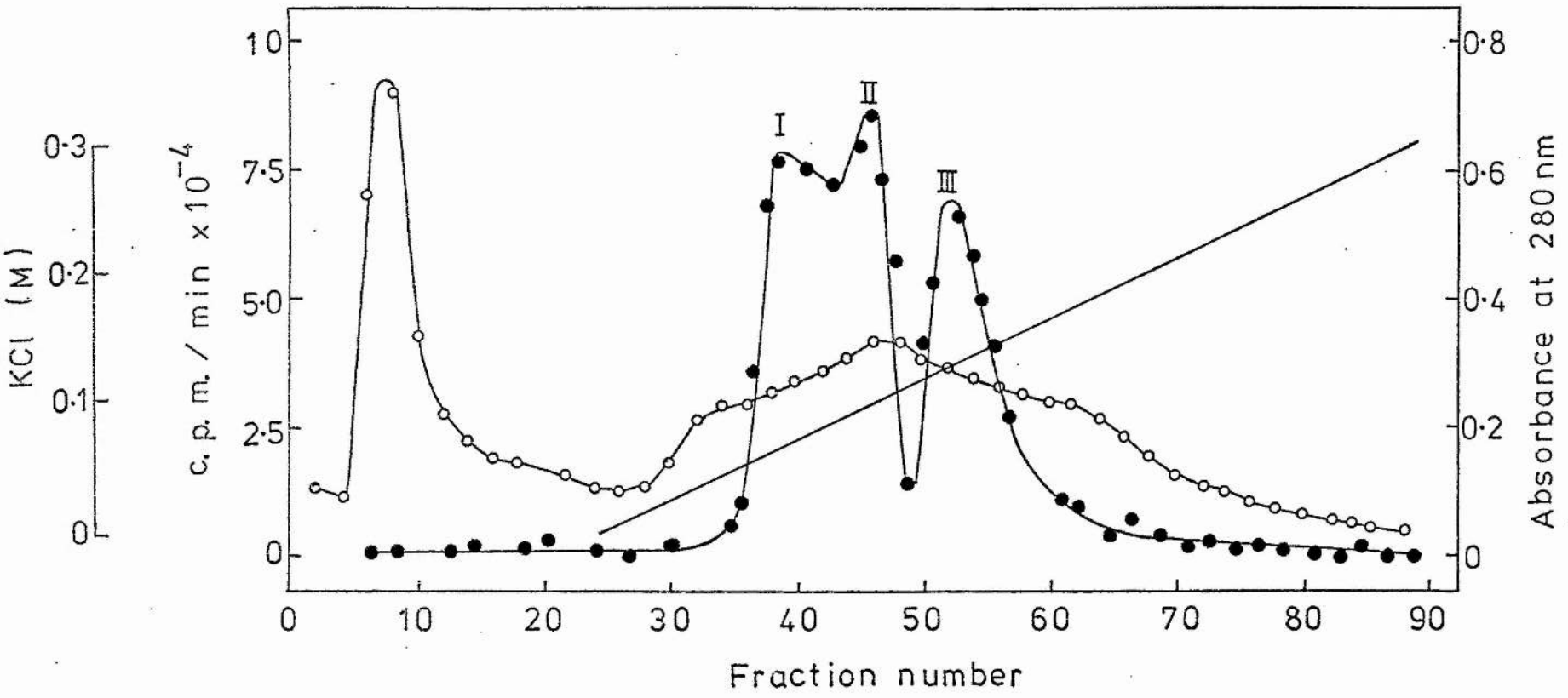

KCl (M)
0·3
0·2
0·1
0
c. p. m. / min x 10⁻⁴
10
7·5
5·0
2·5
0
I
II
III
0·8
0·6
0·4
0·2
0
Absorbance at 280 nm
0
10
20
30
40
50
60
70
80
90
Fraction number

Fig. 44 Distribution of leucine: tRNA ligase activity ●——● and protein O——O following DEAE cellulose fractionation of protein extracted from cells grown for 7 days in M-1D. Protein was eluted from DEAE cellulose with a 0-0.3M KCl gradient.

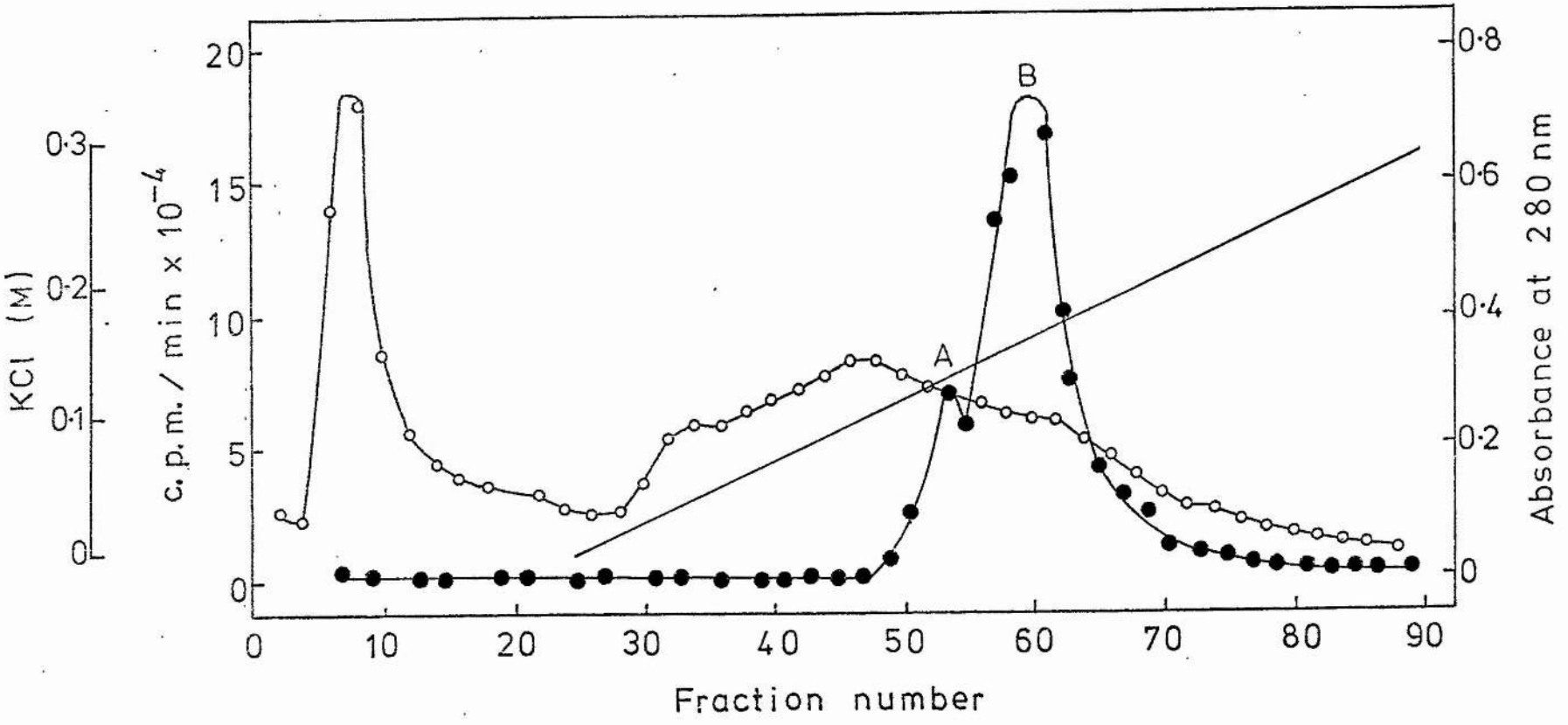

KCl (M)
0·3
0·2
0·1
0
c.p.m. / min x 10^{-4}
20
15
10
5
0
A
B
0·8
0·6
0·4
0·2
0
Absorbance at 280 nm
0
10
20
30
40
50
60
70
80
90
Fraction number

Fig. 45 Distribution of arginine: tRNA ligase activity ●——● and protein O——O following re-chromatography of activity regions I and II (fraction numbers 32 - 49, Fig. 43) on DEAE cellulose. Protein was eluted from DEAE cellulose with a 0.03-0.18M KCl gradient.

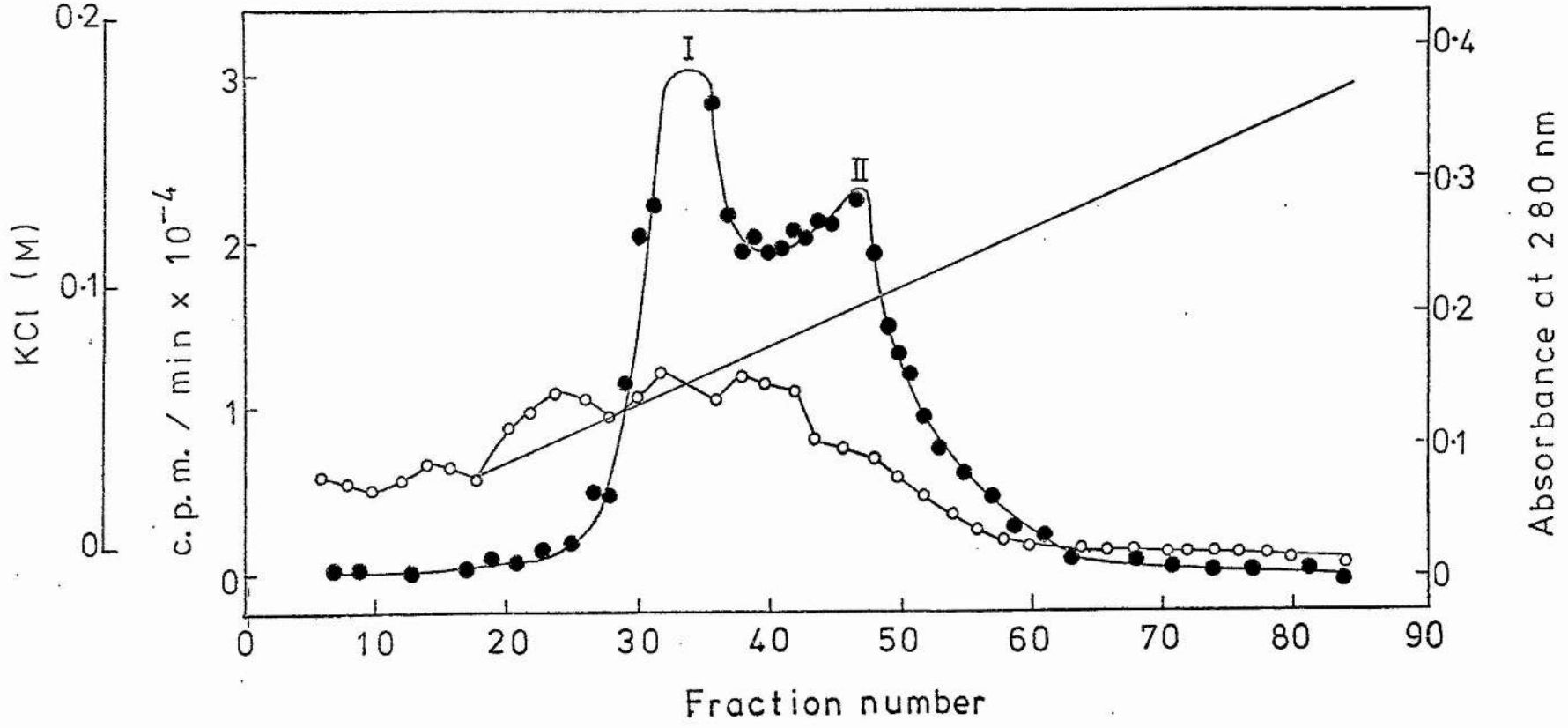
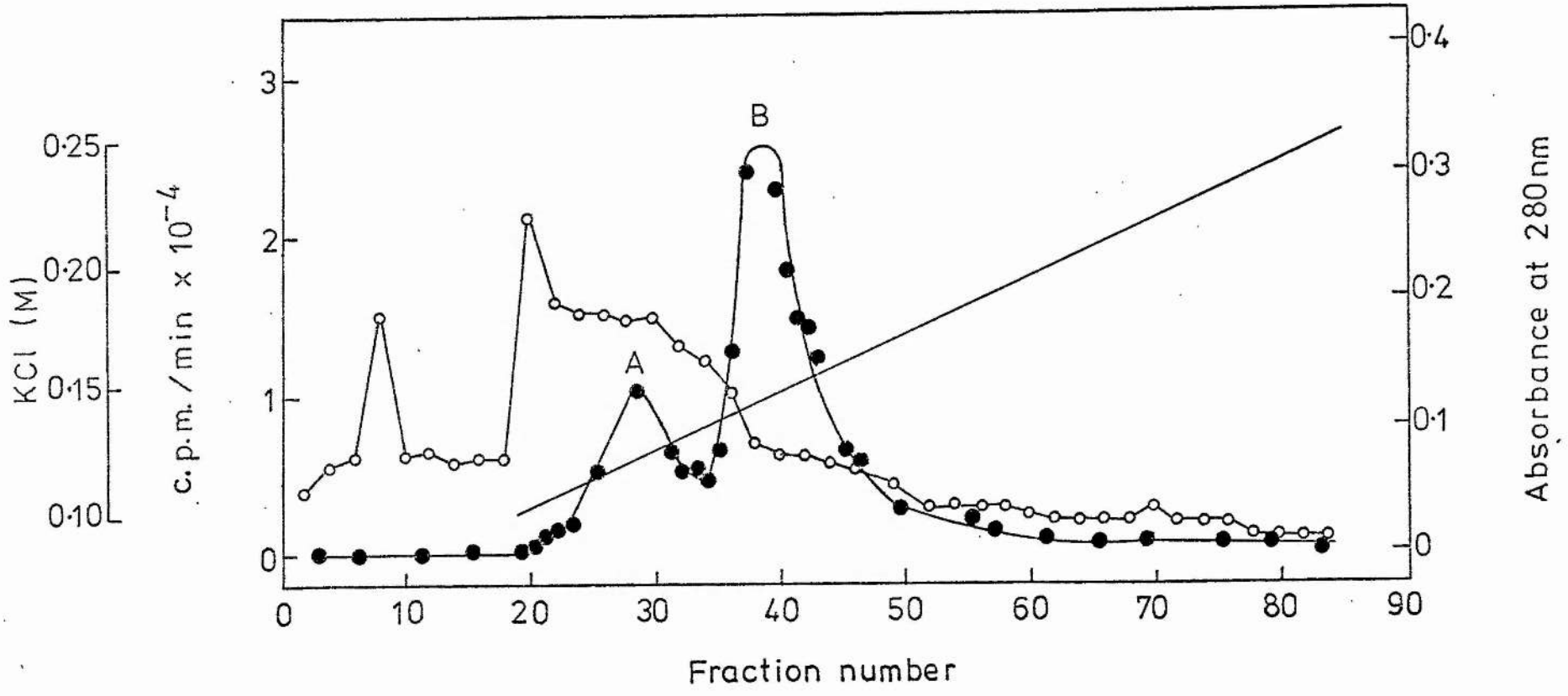

KCl (M)
0·2
0·1
0
c.p.m. / min x 10^{-4}
3
2
1
0
I
II
0·4
0·3
0·2
0·1
0
Absorbance at 280 nm
0
10
20
30
40
50
60
70
80
90
Fraction number

Fig. 46 Distribution of leucine: tRNA ligase activity ●——● and protein O——O following re-chromatography of activity regions A and B (fraction numbers 50 - 65, Fig. 44) on DEAE cellulose. Protein was eluted from DEAE cellulose with a 0.1-0.25M KCl gradient.


KCl (M)
0·25
0·20
0·15
0·10
c.p.m./min x 10^{-4}
3
2
1
0
A
B
0·4
0·3
0·2
0·1
0
Absorbance at 280nm
0
10
20
30
40
50
60
70
80
90
Fraction number

composed of two species of which B predominates.

DEAE Cellulose Chromatography of Enzyme Extracted from Cells Grown for 2 Days in M-1D (Cells in Lag Phase) and Cells Grown for 2 Days in Nitrateless M-1D

The density labelling experiments reported in the previous section utilised 7 day tRNA to assay enzyme activity after isopycnic equilibrium centrifugation. The assay conditions were therefore not homologous. To determine if multiple enzymic species could be detected under these conditions, i.e. during the density labelling studies, protein was extracted from cells that had been grown either for 2 days in M-1D (cells in lag phase) or 2 days in nitrateless M-1D, and fractionated on DEAE cellulose. In all cases protein was eluted with a 0-0.3M linear KCl gradient. The distribution patterns obtained clearly show the presence of three activity regions for arginine: tRNA ligase, both in cells grown for 2 days in M-1D (Fig. 47), and in those grown for 2 days in nitrateless M-1D (Fig. 48). There were similarly two activity regions for leucine: tRNA ligase under both growth conditions (Fig. 49 and Fig. 50).

Under the three separate growth conditions examined, therefore, i.e. cells grown for 7 days in M-1D (cells in exponential phase), for 2 days in M-1D (cells in lag phase) and for 2 days in nitrateless M-1D, three ligase species cognate for arginine and two ligase species cognate for leucine were observed.

It could be argued, however, that the separated species are the result of some artifact of the chromatographic procedure or that they may be dimers or subunits of each other, similar to leucine: tRNA ligase in E. Coli (Rouget & Chapeville, 1970). A more rigorous proof would require re-chromatography of each individual activity region and the demonstration that after re-chromatography the position of the activity region was maintained. The instability of these

Fig. 47 Distribution of arginine: tRNA ligase activity ●——● and protein O——O following DEAE cellulose fractionation of protein extracted from cells grown for 2 days in M-1D. Protein was eluted from DEAE cellulose with a 0-0.3M KCl gradient.

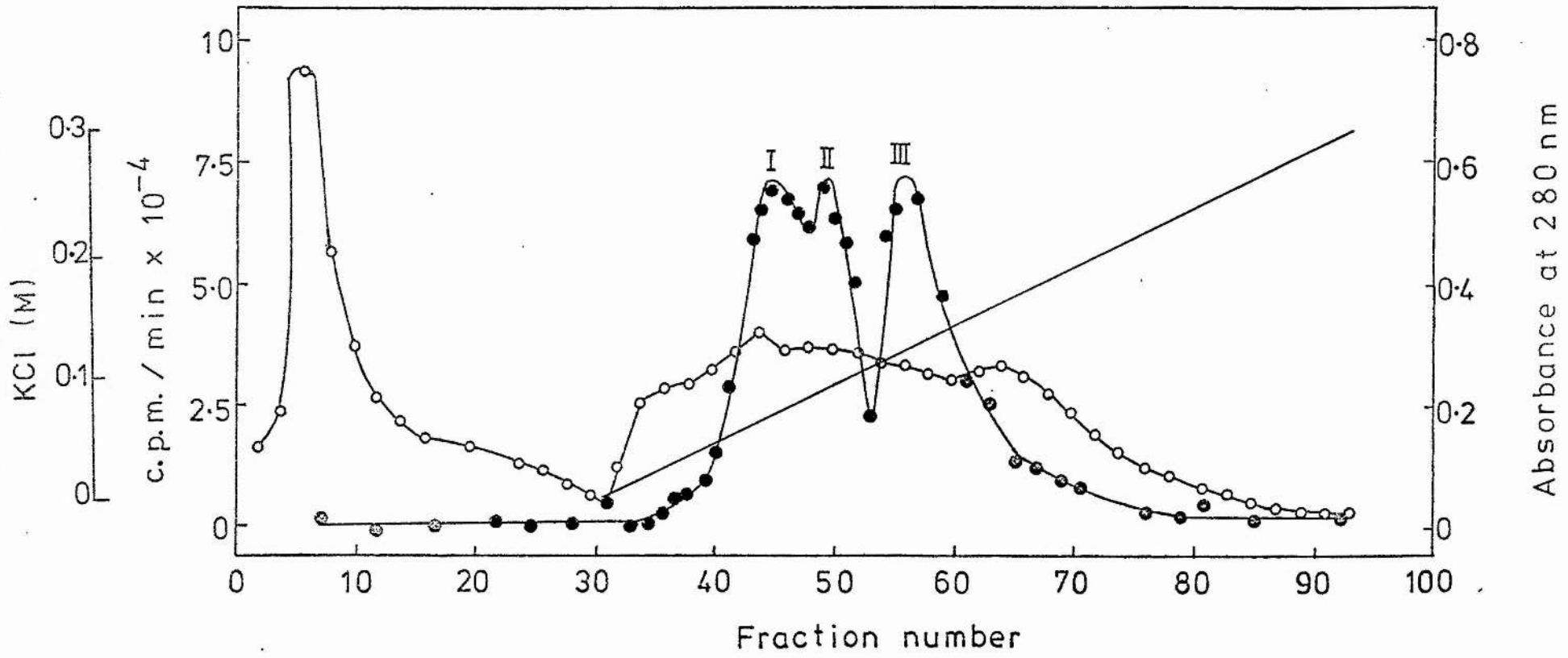

KCl (M)
0·3
0·2
0·1
0
c.p.m. / min x 10^{-4}
10
7·5
5·0
2·5
0
I
II
III
0·8
0·6
0·4
0·2
0
Absorbance at 280 nm
0
10
20
30
40
50
60
70
80
90
100
Fraction number

Fig. 48 Distribution of arginine: tRNA ligase activity ●——● and protein O——O following DEAE cellulose fractionation of protein extracted from cells grown for 2 days in nitrateless M-1D. Protein was eluted from DEAE cellulose with a 0-0.3M KCl gradient.

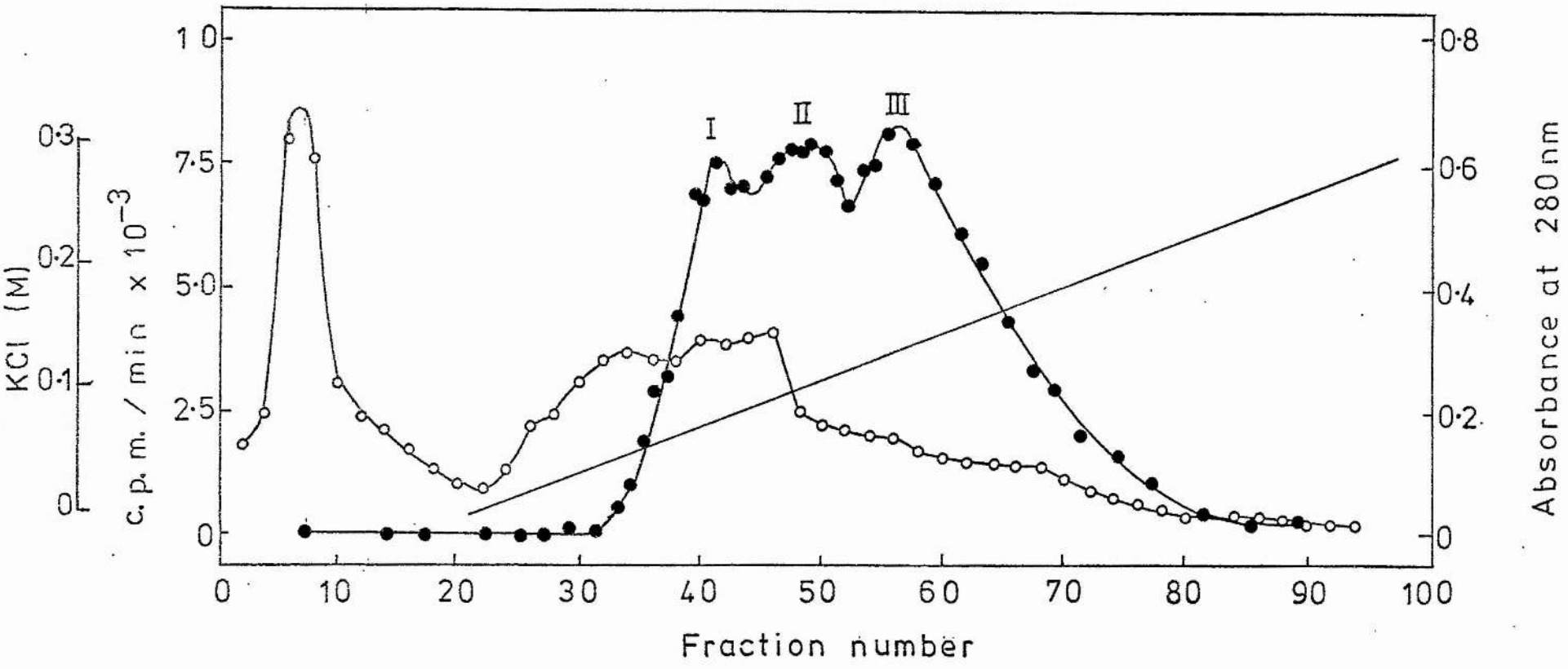

KCl (M)
0·3
0·2
0·1
0
c.p.m. / min x 10⁻³
10
7·5
5·0
2·5
0
I
II
III
0·8
0·6
0·4
0·2
0
Absorbance at 280 nm
0
10
20
30
40
50
60
70
80
90
100
Fraction number

Fig. 49 Distribution of leucine: tRNA ligase activity ●——● and protein O——O following DEAE cellulose fractionation of protein extracted from cells grown for 2 days in M-1D. Protein was eluted from DEAE cellulose with a 0-0.3M KCl gradient.

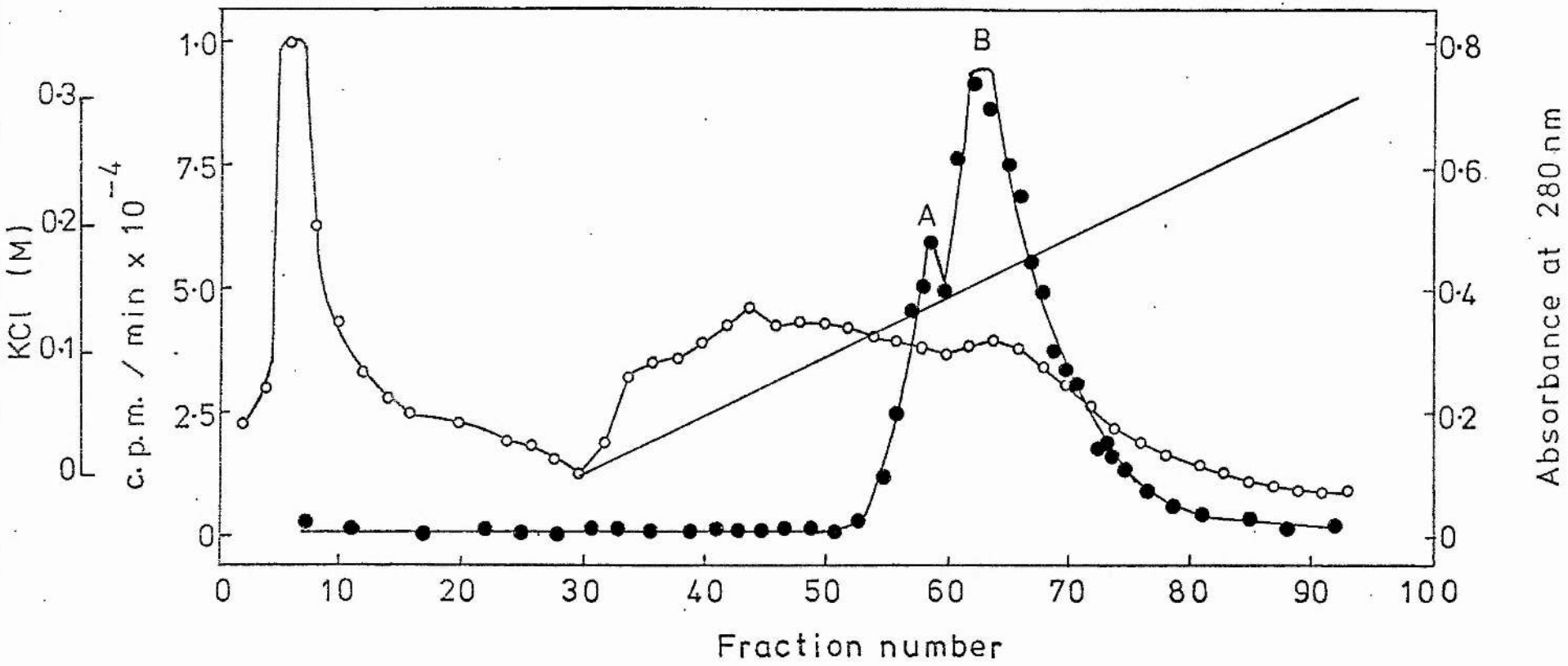
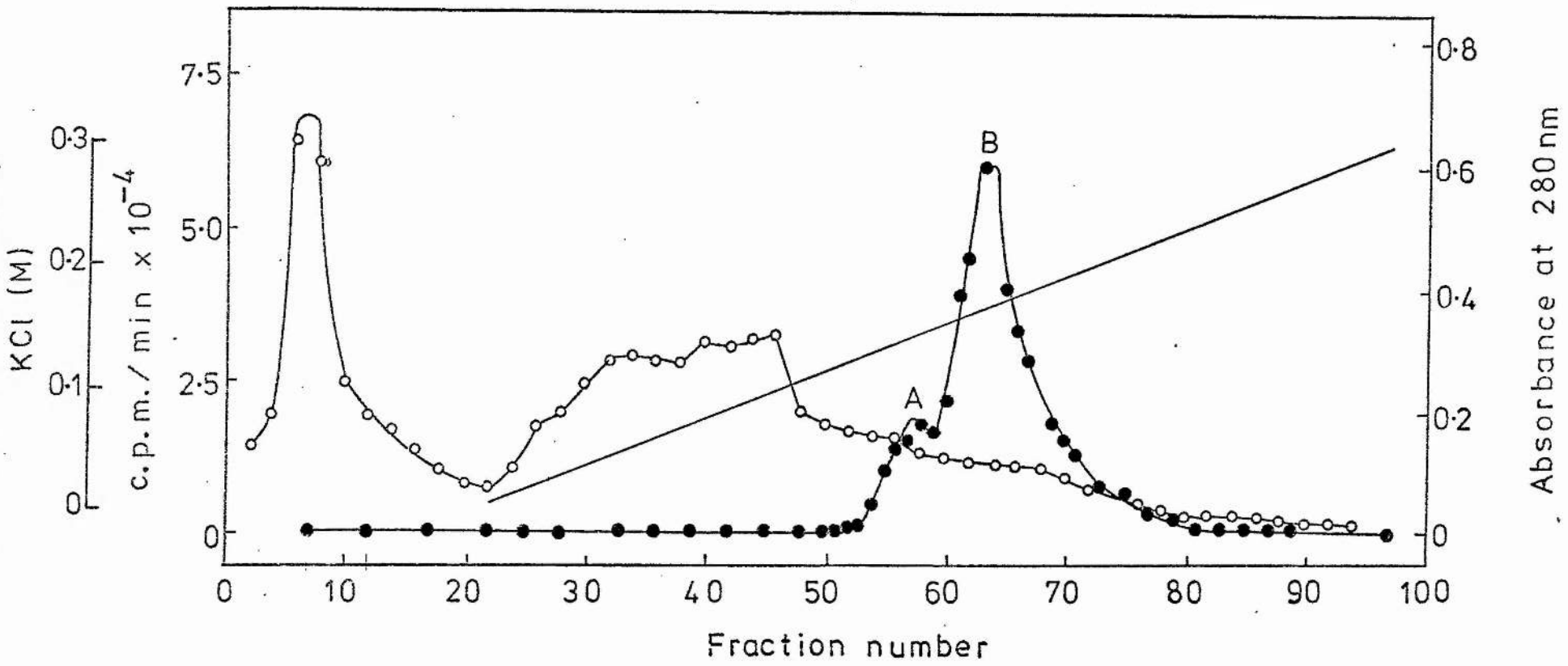

KCl (M)
0·3
0·2
0·1
0
c.p.m. / min x 10⁻⁴
1·0
7·5
5·0
2·5
0
A
B
0·8
0·6
0·4
0·2
0
Absorbance at 280 nm
0
10
20
30
40
50
60
70
80
90
100
Fraction number

Fig. 50 Distribution of leucine: tRNA ligase activity ●—● and protein O—O following DEAE cellulose fractionation of protein extracted from cells grown for 2 days in nitrateless M-1D. Protein was eluted from DEAE cellulose with a 0-0.3M KCl gradient.


KCl (M)
0·3
0·2
0·1
0
c.p.m./min x 10^{-4}
7·5
5·0
2·5
0
A
B
0·8
0·6
0·4
0·2
0
Absorbance at 280 nm
0
10
20
30
40
50
60
70
80
90
100
Fraction number

enzymes, especially to dialysis, (Table 6) makes this difficult to perform.

However, there is some evidence to suggest that the species are truly distinct:-

(1) when the activity regions I and II of arginine: tRNA ligase (Fig. 43) and the two leucine activity regions A and B (Fig. 44) were re-chromatographed with shallower KCl gradients, the activity regions were eluted in the same gradient positions (Fig. 45 and Fig. 46).

(2) re-chromatography of the arginine activity regions I and II (Fig. 45) did not reveal the presence of any activity region III, suggesting it was not derived from regions I or II.

An attempt was made to estimate the relative percentage of each activity region by extrapolating the activity peaks back to the base line, weighing each peak and expressing the weight as a percentage of the total weight of all the peaks (Table 10). Under the three growth conditions examined, the relative proportions of each activity region was fairly constant, with the exception of arginine: tRNA ligase in nitrateless medium where there was either an increase in the amount of region III or a decrease in the amount of regions I and II.

In exponentially growing cells, where assay conditions were homologous, presumably all the enzyme species present in the extraction were detected. However, extracts from cells in lag phase and in nitrateless medium, where assay conditions were not homologous, could contain other ligase species which were not detected because they were unable to recognise 7 day tRNA. Pollock & Filner (1970), using homologous assay conditions, have suggested the presence of an arginine:tRNA ligase species in exponentially growing XD cells which is absent in stationary phase cells. Although we recognise the

Table 10. Percentage distribution of the activity regions, obtained from DEAE cellulose chromatography, of arginine: tRNA ligase and leucine: tRNA ligase, extracted from cells grown for 7 days in M-1D, for 2 days in M-1D and for 2 days in nitrateless M-1D

Arginine: tRNA ligase

% of total activity distribution

Activity region	7 day enzyme	2 day enzyme	2 day nitrateless enzyme
I	37.0	39.4	22.2
II	30.5	26.6	30.9
III	32.6	33.4	46.9

Leucine: tRNA ligase

% of total activity distribution

Activity region	7 day enzyme	2 day enzyme	2 day nitrateless enzyme
A	21.6	27.6	19.9
B	78.4	72.4	80.1

problems involved in using non-homologous assay conditions, they do not affect the observation that in our density labelling experiments there were three ligase species cognate for arginine, which were present in approximately equal proportions, and two ligase species cognate for leucine, of which one predominated. These experiments, therefore, support our previous suggestion that the variable band widths found for arginine: tRNA ligase in the density labelling studies may be due to the presence of multiple enzymic species. A more detailed analysis of how this might occur will be given in the general discussion.

Although our studies have demonstrated, under homologous assay conditions, the presence of three ligase species cognate for arginine, Cowles & Key (1973) were able to detect only a single arginine: tRNA ligase in developing pea roots. However, chromatographically separable arginine: tRNA ligases were found in the chloroplasts and in the cytoplasm of pea seedlings (Aliev & Filippovich, 1968) and ligase species cognate for arginine have been located both in the chloroplasts and in the cytoplasm of Phaseolus vulgaris, which differed in their tRNA specificity and in their ability to exhibit ATP-pyrophosphate exchange in the presence of canavanine (Burkard et al., 1970).

As far as enzymes cognate for leucine are concerned, Anderson & Cherry (1969) have shown that preparations from soybean hypocotyls and cotyledons differed in their ability to acylate iso-accepting leucine: tRNAs. Subsequently, three enzymes cognate for leucine were fractionated from soybean cotyledons, but only two species were observed from the hypocotyls (Kanabus & Cherry, 1971).

In germinating pea seedlings, leucine: tRNA ligase from epicotyls, cotyledons and leaves has been fractionated into two species on hydroxyapetite columns. The relative amounts of these two species varied considerably between the three preparations, due to the existence

of a chloroplast specific species (Wright et al., 1974). More recently, three enzymes cognate for leucine have been reported in Phaseolus vulgaris, by Guillemaut et al.(1975), two of which appeared to be present in chloroplasts, mitochondria and cytoplasm, and one of which was chloroplast specific. The literature would therefore suggest that there are often chloroplast specific amino acid: tRNA ligases. Tobacco XD cells, however, possess no chloroplasts but it is possible that the multiplicity observed for the enzymes cognate for arginine and leucine may be in part due to the existence of mitochondrial or proplastid species.

GENERAL DISCUSSION

In higher plants amino acid: tRNA ligases have only been implicated in protein synthesis, whereas in micro-organisms, these enzymes or their aminoacyl-tRNA products also appear to be involved in important regulatory processes. For example, aminoacyl-tRNA appears to be active as the co-repressor of several amino acid biosynthetic pathways, viz. those for valine (Eidlic & Neidhardt, 1965), isoleucine (Szentirmai et al., 1968), leucine (Alexander et al., 1971) and histidine (Roth & Ames, 1966; Schlesinger & Magasanik, 1964). These investigators have shown that either inhibitors of ligases or mutants with altered or defective ligases causes derepression of the biosynthetic enzymes of the cognate amino acid. This therefore suggests that the level of tRNA acylation is an important regulatory factor.

In other amino acid biosynthetic pathways, however, there is no such correlation between the level of tRNA acylation and repression of amino acid biosynthesis (Ravel et al., 1965; Schlesinger & Nester, 1969; Gross & Rowberry, 1969). In the pathway leading to arginine, the situation appears to be unclear. For example, Hirshfield et al., (1968) could show no derepression of two arginine biosynthetic enzymes in E. coli mutants with defective arginine: tRNA ligases, whereas Williams (1973) has implicated arginine: tRNA ligase in the generation of a repression signal.

At present, therefore, only some ligases appear to be involved in the repression of amino acid biosynthesis, more specifically those of the branched chain and histidine amino acids. More detailed reviews on the regulation of these two pathways are available. (Umbarger, 1969; Goldberger & Kovach, 1972).

In many strains of E. coli, a requirement for amino acid

activation or aminoacyl-tRNA synthesis is also found in the stringent response. Amino acid starvation brings about the stringent response and involves a reduction in the rate of several biochemical processes including RNA synthesis, nucleotide synthesis and protein turnover (Pardee & Prestidge, 1956; Cashel & Gallant, 1969; Haseltine & Block, 1973). One or more of the aminoacyl-tRNA pools appear to be involved in this response, rather than the intracellular levels of amino acids themselves, since inactivation of any of the ligases elicits the stringent response even in the presence of all the amino acids (Neidhardt,1966). During the stringent response the accumulation of the nucleotides ppGpp and pppGpp is observed. They are synthesised on the ribosomes (Haseltine et al., 1972) and produced by a reaction which is normally involved in protein synthesis but which idles during the stringent response (Cashel & Gallant, 1969). The signal that triggers this idling reaction appears to be the absence of a single species of aminoacyl-tRNA at the acceptor site of a ribosome bound to mRNA (Haseltine & Block, 1973).These nucleotides have also been shown to inhibit ribosome synthesis in cell free systems (Goldberg et al., 1974).

However, even in relaxed strains where the stringent response is not observed, amino acid: tRNA ligases are still important, since by controlling the availability of aminoacyl-tRNA species, they must also regulate protein synthesis <u>per se</u>. This of course is true for all living cells.

There is also considerable evidence that cellular differentiation is regulated, at least in part, at the level of mRNA. It is possible, therefore, that both the concentration of specific tRNA molecules and of their cognate amino acid: tRNA ligases may have important roles in modulating translation of mRNA.

This is supported by the observations that ligases undergo changes during fundamental biological processes, such as growth and development (Henshall & Goodwin, 1964; Hinde et al., 1966; Anderson & Fowden, 1969; Cowles & Key, 1973; Norris et al., 1973; Wray et al., 1974), and senescence (Bick & Strehler, 1971; Nathan & Richmond,1974); that, consistent with our studies, there are often multiple ligases cognate for a particular amino acid (Kanabus & Cherry, 1971; Cowles & Key, 1972, 1973; Wright et al., 1974; Guillemaut et al., 1975), which may change during growth and development (Cowles & Key, 1973); and that there are multiple iso-accepting tRNA species for an amino acid, whose levels may also change during growth and development (Vold & Sypherd, 1968; Anderson & Cherry, 1969; Bick et al., 1970).

Although many models have been proposed to explain how both tRNA and ligase may be involved in the modulation of mRNA translation (Sueoka & Kano-Sueoka, 1970) there is as yet little in vivo evidence to support any of them.

The fact that these enzymes appear to be involved in such fundamental regulatory processes would indicate that it is important for the cell to maintain a tight and co-ordinate control of these enzymes. This made the study of their regulation of great interest, particularly in respect of plant tissues, where very little is known.

Suspension culture was chosen for these studies in preference to the intact plant because it allows experimentation with a more or less homogenious cell population, the culture is aseptic, thus avoiding the problems of bacterial contamination, and perhaps of more importance in relation to our studies, the use of suspension culture allows manipulation of the culture medium, enabling cells to be grown under any condition at will.

The regulation of these enzymes has been most widely studied in micro-organisms and several modes of regulation have been proposed, namely

(1) metabolic regulation

(2) regulation by energy charge

(3) regulation by amino acid biosynthetic intermediates

(4) regulation by amino acid or some derivative

(1) Metabolic regulation

The concept of metabolic regulation was first introduced by Parker & Neidhardt (1972). These investigators have shown a coupling of ligase formation to growth rate in E. coli and Salmonella typhimurium grown in media of differing richness. Over a 7 fold range in growth rate, the levels of arginine: and valine: tRNA ligases changed by a factor of 2.5 (Parker & Neidhardt, 1972). By use of immunological and isotopic labelling techniques, it was shown that the increase in activity observed in enriched medium was due to de novo synthesis and that degradation of enzyme protein appeared to be insignificant. Even in slow growing cultures, the low activity was not caused by specific degradation (Parker et al., 1974). Results consistent with some form of metabolic regulation in micro-organisms have been demonstrated by other investigators, (Hirshfield & Zamecnik, 1972; Anderson & Neidhardt, 1972; Hirshfield et al., 1975; Cassio et al., 1975), although this does not always seem to be the case (Cassio et al., 1970; Anderson & Neidhardt, 1972). There is little evidence for metabolic regulation of amino acid: tRNA ligases in higher plants, but in our studies, the observation that ligase activity remains constant in nitrateless M-1D (Fig. 15) but undergoes a 7 fold increase in activity when stationary phase cells are transferred to fresh M-1D medium (Fig.14)

may indicate the existence of a similar regulatory mechanism.

Verma & Marcus (1974) have recently reported that when stationary phase cell culture of Arachis hypogaea is diluted into fresh medium, there is a 10 fold increase in protein synthesis which is accompanied by a 2-4 fold increase in polyribosome content as well as 3-4 fold increase in the rate of mRNA synthesis. Similar observations have been made during germination of wheat embryos (Obendorf et al., 1974). It is possible, therefore, that the increases in activity of arginine: and leucine: tRNA ligases following transfer to fresh M-1D medium, may reflect an increase in the availability of transcription/translation machinery. In other words, the enzymes are synthesised constitutively.

This is contrary to what is found in a number of highly differentiated tissues. For example, in the silk-gland of Bombyx mori (Garel et al., 1970), lens tissue (Garel et al., 1971) and reticulocytes (Smith & McNamara, 1971), the relative amounts of each free tRNA are directly related to the amino acid composition of the proteins synthesised in that tissue (i.e. fibroin and sericin, crystallins, haemoglobin). More recently, Chavancy et al., (1975) have shown that amino acid: tRNA ligases, like tRNAs, are quantitively related to fibroin synthesis in the silkgland of Bombyx mori. It would appear, therefore, that in these tissues there is a strict and co-ordinated regulation of the concentration of tRNAs and ligases, adjusted to the amino acid composition of the protein synthesised in that tissue.

(2) Regulation by energy charge

In Salmonella typhimurium, arginine:, histidine:, lysine: and valine: tRNA ligases are inhibited by ADP and AMP (Brenner et al., 1970), suggesting that these enzymes may be regulated by the energy charge of the cell. The concept of energy charge was first

introduced by Atkinson & Walton (1967) who defined energy charge as the ratio of the concentration of ATP plus one half the concentration of ADP, to the total adenine nucleotide concentrations, i.e. Energy charge = $\frac{[(ATP) + \frac{1}{2}(ADP)]}{[(ATP) + (ADP) + (AMP)]}$ and considered the adenylate system analogous to an electrochemical storage cell in its ability to accept, store and supply chemical energy. Thus the system AMP + 2P $\rightleftharpoons$ ATP + $2H_2O$ is fully charged when all adenylate is in the form of ATP, and discharged when only AMP is present. Therefore cellular reactions involving ATP utilisation (such as synthesis of aminoacyl-tRNA) might be expected to be inhibited by AMP and ADP, whilst ATP would inhibit energy generating systems. In both micro-organisms and animal systems, a large number of enzymes appear to be regulated by energy charge (Atkinson, 1968; Atkinson, 1969; Chapman et al., 1971) but although this regulatory mechanism has been shown to occur in higher plants (Weissman, 1972; Ching & Ching, 1972), it is not well documentated and there is no evidence for its role in regulating amino acid: tRNA ligases, either in tobacco XD cells or in any other plant system.

In common with other investigations, however, (Novelli, 1967; Hall & Tao, 1970; Burkard et al., 1970; Cornelis & de Patoul, 1975) we have shown that the activities of both arginine: and leucine: tRNA ligases <u>in vitro</u> are very much dependent on the ATP/Mg^{2+} ratio (Fig. 7) and that the optimal ratio required for arginine: tRNA ligase activity is different to that required for leucine: tRNA ligase. Therefore, the marked dependence of ligase activity on these levels raises the question of whether ATP and Mg^{2+} are likely to influence the activity of these enzymes <u>in vivo</u>. It must be recognised that our experiments have involved bulk enzyme extractions which, as we have shown, contain multiple

ligases (Figs. 43 - 50), derived not only from the cytoplasm, but perhaps also from subcellular organelles. It is conceivable, however, that at the sites of protein synthesis the Mg^{2+} and ATP levels would be similar, such that all ligases could not be operating maximally at the same time. The possibility, therefore, exists that Mg^{2+} and ATP levels may be important factors in regulating amino acid tRNA ligases.

(3) Regulation by amino acid biosynthetic intermediates

In both E. coli (Yem & Williams, 1971) and Neurospora crassa (Nazario, 1967), arginine: tRNA ligase is inhibited by intermediates of the biosynthetic pathway leading to arginine. In Neurospora, argininosuccinate lyase mutants accumulate endogenously synthesised argininosuccinate and in addition have high levels of ornithine transcarbamylase, even in the presence of excess arginine. It was found that arginine: tRNA ligase was inhibited by the accumulated argininosuccinate and that this led to a marked reduction in the percentage of charged arginyl-tRNA. This, therefore, is not only a mechanism whereby arginine: tRNA ligase can be regulated but also provides further evidence for the involvement of arginine: tRNA ligase in the regulation of arginine biosynthesis. In higher plants, however, there appears to be no report of amino acid: tRNA ligases being regulated by amino acid biosynthetic intermediates.

(4) Regulation by amino acids or some derivative

In micro-organisms, it is well documented that a number of amino acid: tRNA ligases are regulated by repression-derepression mechanisms. For example, density labelling studies have revealed that restriction of the supply of a particular amino acid specifically increases the rate of de novo synthesis of the cognate ligase (Williams & Neidhardt, 1969; Archibold & Williams, 1972;

McGinnis & Williams, 1972a). In a few cases, it is aminoacyl-tRNA which appears to be involved in the repression of amino acid: tRNA ligase. This has been reported for histidine: tRNA ligase in Salmonella typhimurium (McGinnis & Williams, 1972ab) and recently for methionine: tRNA ligase in E. coli (Cassio, 1975).

It would appear, therefore, that in some cases there is a common regulatory element in both the control of amino acid biosynthesis and amino acid: tRNA ligase synthesis. Jackson et al., (1974), for example have presented evidence for altered repression control of synthesis of both the branched chain amino acid biosynthetic enzymes and cognate ligases in E. coli. In other studies, however, derepression of amino acid biosynthetic enzymes has not been accompanied by a corresponding derepression of ligases (Roth & Ames, 1966). It has been suggested that whereas the co-repressors of the two regulatory processes may be common, the apo-repressors are different. (McGinnis & Williams, 1972ab; Archibold & Williams, 1972).

In the studies reported in this thesis, we have looked for, but have been unable to find any evidence for a role of amino acids in the regulation of amino acid: tRNA ligases in tobacco XD cells. Amino acid restriction, by growing cells in nitrateless M-1D medium, did not result in a derepression of arginine: or leucine: tRNA ligases (Fig. 15). There was also no evidence for derepression of arginine: tRNA ligase, similar to that found for the valine enzyme in yeast (Ehresmann et al., 1971), when cells that had been grown for 50 generations in M-1D supplemented with arginine were transferred to normal M-1D (Fig. 16).

Even in micro-organisms the situation is far from clear. Coleman & Williams (1974) have recently shown an involvement of the first enzyme in histidine biosynthesis in the repression of

histidine: tRNA ligase in E. coli. Moreover, the acceleration of ligase formation at growth conditions that restrict the supply of an amino acid is not always observed and the way in which the restriction is imposed and the nature of the bacterial strain used seems to have a pronounced effect on the response (Nass & Neidhardt, 1967; Dale & Nester, 1971; Gahr & Nass, 1972).

In contrast, however, to the well established regulatory mechanisms of gene expression found in micro-organisms, (Jacob & Monod, 1961), little is known about control of gene action in higher plants and although models have been proposed which involve regulatory genes (Britten & Davidson, 1969), similar to those found in micro-organisms, no true repression mechanisms have yet been identified.

Many plant enzymes, such as those involved in amino acid biosynthesis (Miflin, 1973) are regulated by end product inhibition and other enzymes are subject to allosteric activation/ inactivation phenomena. In recent years a number of studies have obtained evidence for turnover of total cell protein in higher plants (Huffaker & Peterson, 1974), similar to the observations made in animal systems (Schimke, 1969), but only a few specific enzymes have been shown to be synthesised de novo and to turn over (Table 2).

In this respect, an enzyme which has been studied in great detail is nitrate reductase in tobacco XD cells (Zielke & Filner, 1971). Using the density labelling technique, in which pre-existing protein was labelled with ^{15}N, these authors have demonstrated that this enzyme is under constant turnover, being continuously synthesised and degraded during induction by nitrate, during the period of constant activity after induction, and during decay of enzyme activity in non-inducing conditions.

By additionally labelling pre-existing proteins with ^{14}C- arginine and newly synthesised proteins with ^{3}H- arginine, they were able to show the existence of a large pre-existing ^{15}N amino acid precursor pool, and that both pre-existing protein and newly synthesised protein turned over. Released ^{15}N amino acids were found to be re-utilised for synthesis of nitrate reductase making turnover rates difficult to measure.

In our studies, we have shown that both arginine: and leucine: tRNA ligases are synthesised de novo, not only as they increase in activity (Fig. 29 and Fig. 33) but also when activity remains constant in nitrateless M-1D (Fig. 36). Since there was no increase in activity in nitrateless M-1D, yet both enzymes were being synthesised de novo, degradation must also have been occurring in nitrateless M-1D and therefore both enzymes turned over.

In normal M-1D, the constant band widths of the equilibrium centrifugation profiles at all times after transfer to light medium indicated that leucine: tRNA ligase also turned over as it increased in activity (Table 7). However, such a conclusion could not be made for the arginine enzyme as the band widths were not constant (Table 8). DEAE cellulose chromatography revealed the presence of three ligase species cognate for arginine and two species cognate for leucine. The observation that the arginine species were in approximately equal proportions, whereas one of the leucine species accounted for 80% of the leucine: tRNA ligase activity (Table 10), suggested that the variable band widths observed for arginine: tRNA ligase could be due to this multiplicity rather than to the absence of degradation. For example, the actual buoyant density and band width that is observed in our experiments after transfer to light medium will

be determined from an average of the relative proportions of labelled and unlabelled molecules present in each individual enzyme species. The fact that the three arginine species are in approximately equal proportions means changes in the proportions of labelled and unlabelled molecules in any one species would have a profound effect on the buoyant density and band width that is observed. In contrast, one of the leucine species (activity region B) accounts for 80% of the total leucine: tRNA ligase activity and therefore the observed change in buoyant density and band width will depend predominantly on changes in this species, while the contribution of the other species (activity region A) will have little effect.

If, for example, one of the arginine species was mitochondrial in origin and lighter unlabelled amino acids did not equilibrate with its amino acid precursor pool until 24h after transfer into light medium, then the increase in band width obtained at 24h (Table 8) could be a consequence of an increase in lighter enzyme molecules derived from the mitochondria. However, if this was the case, it might be expected that the increase in band width would be accompanied by a large decrease in buoyant density, but this is not observed (Table 8). If, on the other hand, the three arginine species were synthesised from the same amino acid precursor pool and were turning over at different rates, then presumably at a particular point in time, the average rate of degradation of the three species could be very slow. If this was the case at 24h when enzyme is increasing in activity and being synthesised de novo, a large increase in lighter unlabelled molecules could occur with a resulting increase in band width. Therefore, based on the DEAE cellulose chromatography data and the fact that arginine: tRNA ligase turns

over in nitrateless M-1D, it seems reasonable to conclude that the enzyme also turns over in normal M-1D as it increases in activity.

It was not possible to give a quantitative treatment of the turnover of arginine: and leucine: tRNA ligases from the rate at which the buoyant density decreased with time. This was because of the re-cycling of total cell protein with the resultant re-incorporation of some (heavy) labelled amino acids into enzyme protein synthesised after transfer to light medium (Zielke & Filner, 1971).

A number of investigators, however, have used percent return kinetics to obtain a qualitative comparison of rates of turnover i.e. by measuring the rate of percent return to the unlabelled state. This is given by the equation:-

$$\text{percent return} = \frac{\rho(\text{FL}) - \rho(t)}{\rho(\text{FL}) - \rho(\text{UL})} \times 100$$

where ρ = density, FL = fully labelled enzyme, UL = unlabelled enzyme and t = time after transfer to light medium.

If there is no degradation of enzyme protein then obviously the mean density approaches that of the unlabelled state at a rate dependent entirely on synthesis. However, if, as in the case of arginine: and leucine: tRNA ligases, there is also degradation, the rate at which the buoyant density approaches the buoyant density of the unlabelled state is determined by both synthesis and degradation. This is because the mean density is a measure of the proportions of unlabelled and labelled molecules in the population at any one time, which is also a function of the relative rates of synthesis and degradation. Such percent return plots of our data show that in both M-1D

(Fig. 51) and in nitrateless M-1D (Fig. 52), arginine: and leucine: tRNA ligases turn over at different rates. Leucine: tRNA ligase initially turns over at a much faster rate than the arginine enzyme, whereas the rate of turnover of arginine: tRNA ligase appears to increase between 48h and 72h. Where appropriate, the points in Fig. 51 and Fig. 52 represent the mean values from two experiments.

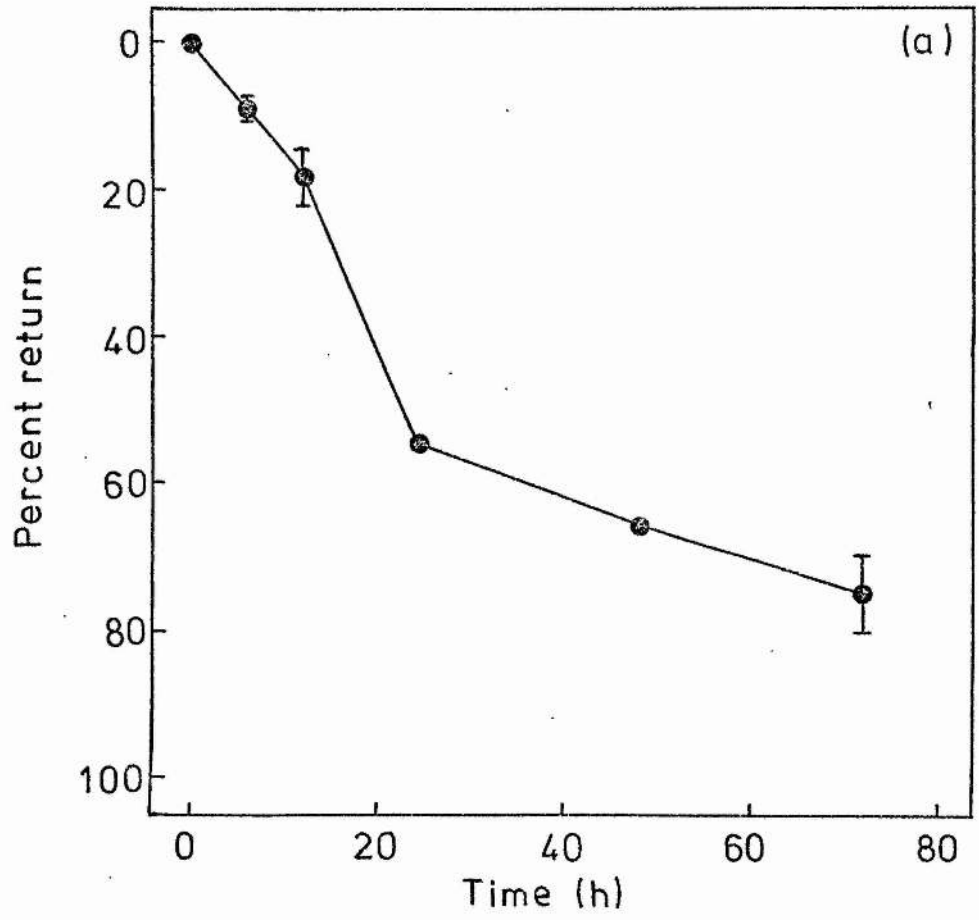
A comparison of these plots, however, is invalid if

(1) the rate of equilibration of unlabelled amino acids with the amino acid precursor pool is rate limiting, since in this situation the rate at which the mean density approaches the unlabelled state would be determined by the rate at which the amino acid precursor pool equilibrates with light unlabelled amino acids.

(2) if the enzymes being compared are synthesised from different amino acid precursor pools, since pool sizes may be different and subject to different effects of re-cycling of total cell protein.

In the case of arginine: tRNA ligase, both in M-1D (Fig. 34 and Table 8) and in nitrateless M-1D (Fig. 38 and Table 9b) there was no initial drop in buoyant density after transfer to light medium. Synthesis, therefore, would appear to be exclusively from heavy labelled amino acids, indicating that the rate of equilibration of light amino acids with the precursor pool is rate limiting for the density shift. However, as we have previously pointed out, leucine: tRNA ligase does show an initial decrease in buoyant density after transfer to both M-1D (Fig. 30 and Table 7) and nitrateless M-1D (Fig. 37 and Table 9a). It can be concluded, therefore, that either equilibration of light amino acids with the precursor pool is not rate limiting or the two

Fig. 51 Kinetics of percent return to the unlabelled state for (a) leucine: tRNA ligase and (b) arginine: tRNA ligase following transfer of 2 day old cells from heavy (labelled) M-1D into light (unlabelled) M-1D.


(a)
Percent return
0
20
40
60
80
100
0
20
40
60
80
Time (h)

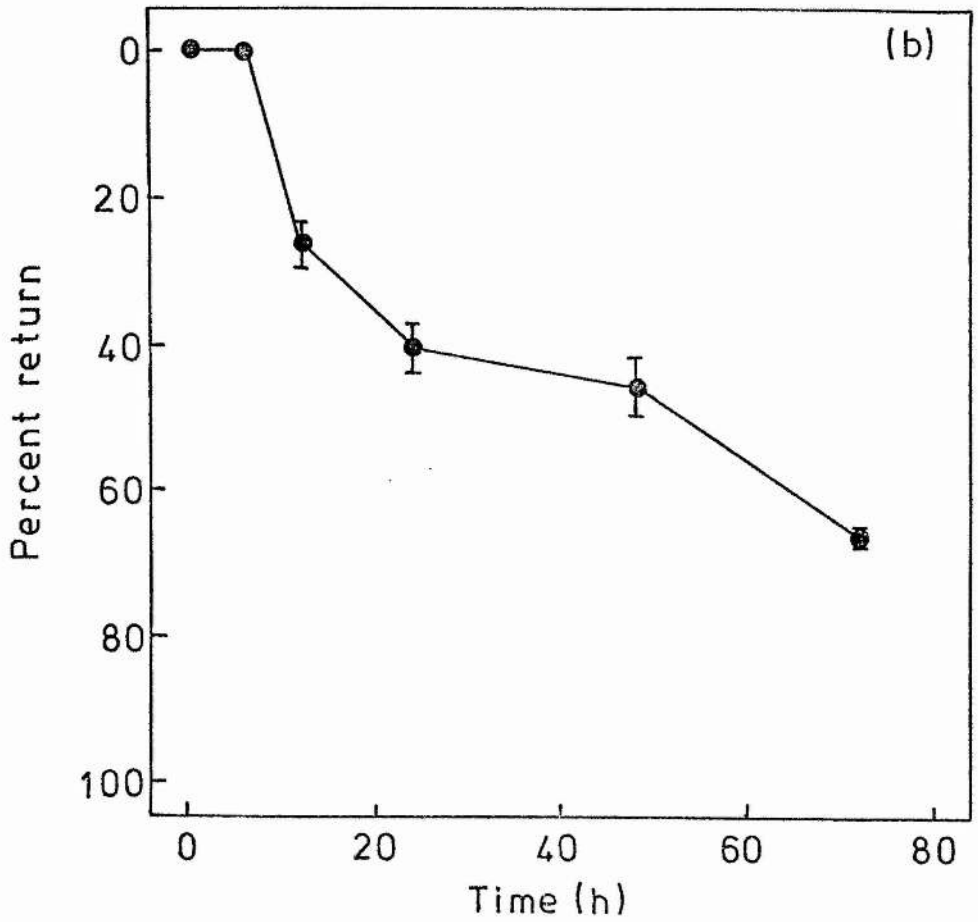
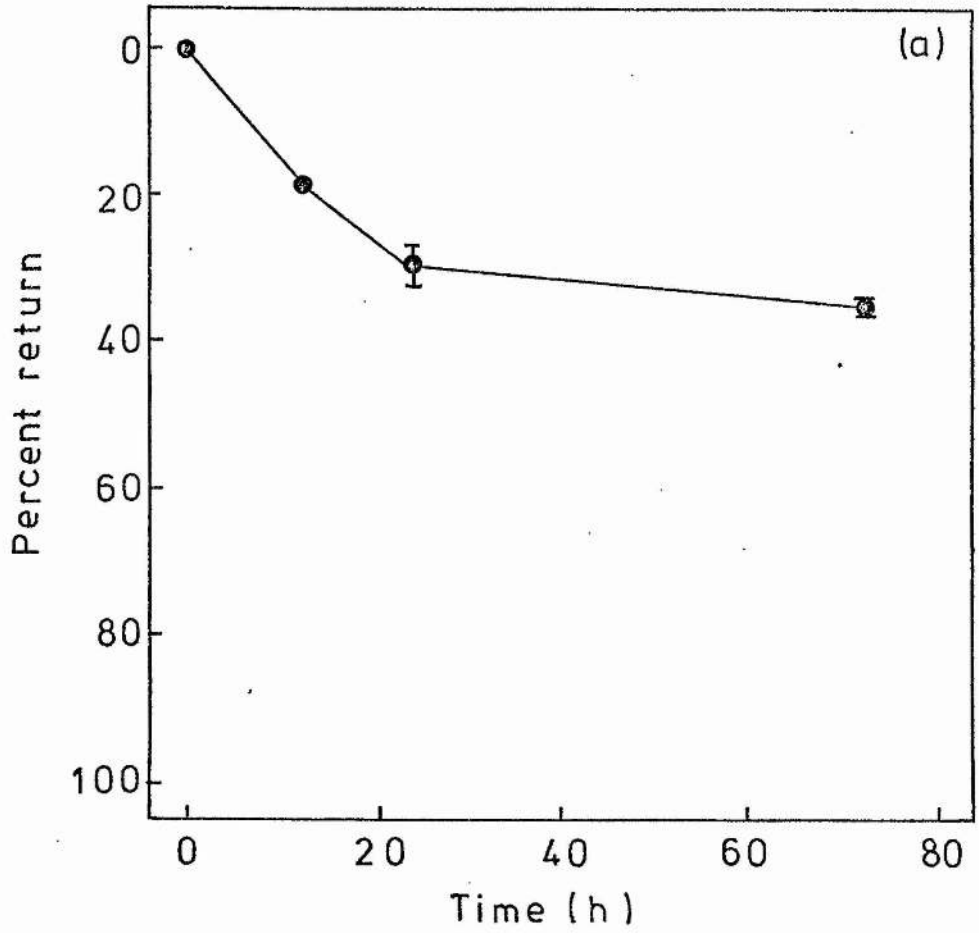
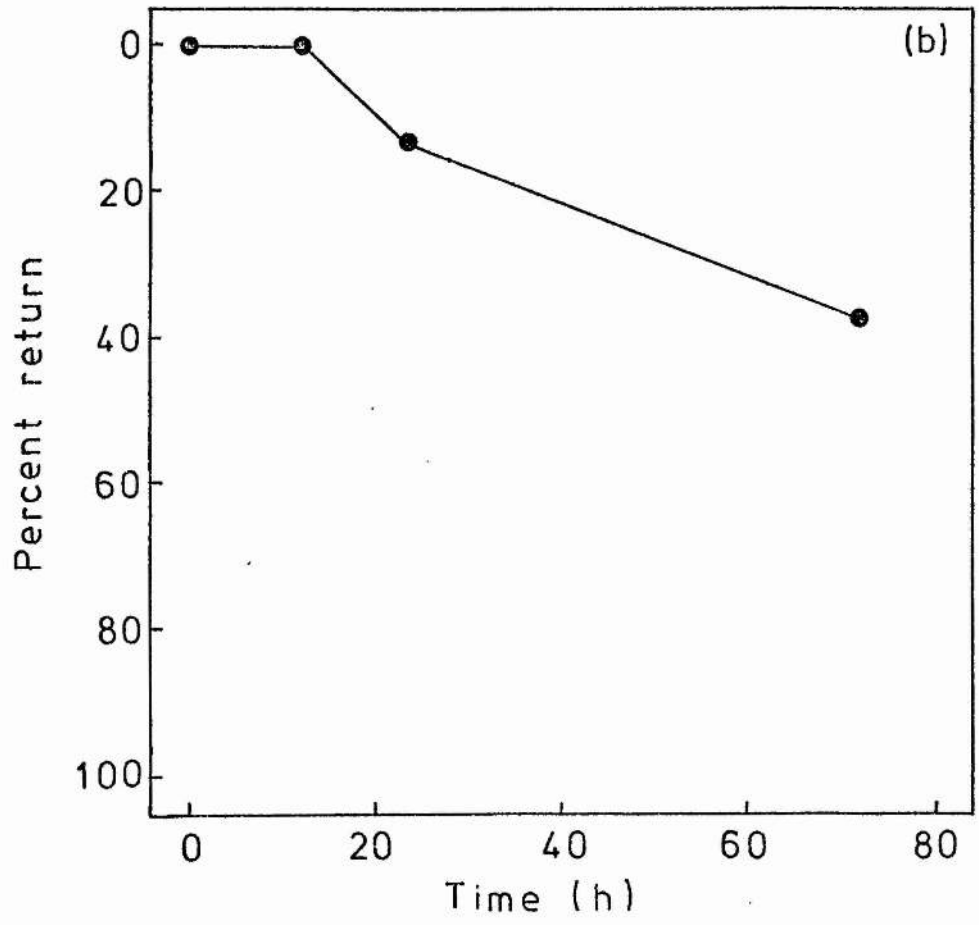

(b)
Percent return
0
20
40
60
80
100
0
20
40
60
80
Time (h)

Fig. 52 Kinetics of percent return to the unlabelled state for (a) leucine: tRNA ligase and (b) arginine: tRNA ligase following transfer of stationary phase cells from heavy (labelled) M-1D into light (unlabelled) nitrateless M-1D.


(a)
Percent return
0
20
40
60
80
100
0
20
40
60
80
Time (h)


(b)
Percent return
0
20
40
60
80
100
0
20
40
60
80
Time (h)

enzymes are synthesised from different amino acid pools. The observation that the buoyant density of arginine: tRNA ligase did not initially decrease after transfer to light medium under two different growth conditions i.e. when two day old cells were transferred to light M-1D and stationary phase cells were transferred to light nitrateless M-1D, would suggest that the two enzymes were in fact synthesised from different amino acid pools. This of course does not preclude the possibility that equilibration of light amino acids with the precursor pool of arginine:tRNA ligase is rate limiting. It would appear, therefore, that our percent return plots are not comparing turnover rates. In a similar study involving two catalase isozymes, V^4 and Z^4 (Quail & Scandalios, 1971), no initial drop in the buoyant density was observed for the V^4 isozyme until 6h after transfer to light unlabelled medium. These investigators totally ignored this fact and assumed that the isozymes were synthesised from the same precursor pool. For reasons discussed above, this cannot be the case, unless of course the two isozymes had vastly different amino acid compositions.

It is clear, therefore, that the interpretation of our results is made difficult by the re-cycling of total cell protein, the existence of multiple enzymic species and complex compartmentation of amino acids.

In Zielke & Filner's (1971) studies on nitrate reductase, heavy labelled stationary phase tobacco XD cells were transferred into light M-1D when there was little enzyme synthesis occurring, whereas in our studies, cells were transferred to light M-1D 48h into the growth cycle when there was already considerable synthesis of ligase occurring. Therefore, the problems that they experienced in relation to equilibration of amino acid precursor pools and

re-cycling of total cell protein would be increased in our studies.

Complex compartmentation of amino acids is particularly common to higher plants (Mclennan et al., 1963; Oaks & Bidwell, 1970). For example, active and inactive leucine pools have been identified in maize root tips (Oaks, 1965), and in soybean hypocotyls there are small leucine and valine protein precursor pools but much larger inactive pools (Holleman & Key, 1967). Thus immediate precursor pools of amino acids may not be in equilibrium with stored amino acids. Recently, in the liver of male Charles rats, Airhart et al. (1974) observed that a common intracellular amino acid pool was not the sole precursor of aminoacyl-tRNA and proposed a model in which there is a functional interaction between amino acid: tRNA ligases and some components of the cell membrane system, and that amino acids within the membrane are activated for protein synthesis regardless of whether they are entering or leaving the cell. The fact that the extent of charging of tRNA seems to co-ordinate a number of critical metabolic activities makes membrane bound ligases an attractive model from the point of view of hormonal interaction and regulation of basic cell function.

A number of investigators have tried to overcome the problems of complex compartmentation and re-cycling of total cell protein when studying protein turnover. For example, Hellebust & Bidwell (1964) used a method similar to constant infusion of radio-isotope. Wheat seedlings were supplied with $^{14}CO_2$ in light for 11½h each day for 6 days in order to label all the possible precursors of protein carbon to the same extent. Turnover rates were measured by the amount of radioactivity accumulated into protein. Oaks (1965) showed for maize that when incorporation of labelled precursor into protein was linear, the precursor pool was saturated and the newly labelled protein had the same specific activity as that of

the precursor pool. Therefore by swamping the precursor pool with valine and leucine at concentrations above which there was no uptake, Holleman & Key (1967) were able to determine the specific activity of the protein precursor pool in soybean hypocotyls and thus determine the turnover. A similar technique has been used for banana (Sacher, 1966). However, swamping a tissue with large amounts of amino acid may cause enzyme inhibitions, amino acid antagonisms and disruption of metabolic pathways (Joy & Folkes, 1965).

Trewavas (1972), using Lemna minor, overcame the problem of metabolic pools by recognising that the immediate precursor of protein is not the free amino acid, but amino acid attached to tRNA. Using either constant infusion or pulse labelling with ^{3}H methyl methionine, he was able to follow the specific activity of the methionine pool contributing to protein synthesis by isolating methionyl-tRNA. If Lemna was grown to isotopic equilibrium on $[^{35}SO_4]$, determination of the ^{3}H:^{35}S ratio in isolated protein gave a direct measurement of the specific activity of the protein and therefore the rate of turnover could be determined.

The disadvantage of this method is that it is difficult technically to perform.

The problems of re-cycling and complex compartmentation have largely been eliminated in the method used by Humphrey & Davies (1975). This method, which has been described in the introduction, involves incubating tissue for a short period on a growth medium containing $^{3}H_2O$. During this time ^{3}H exchanges with the α-carbon atom of most amino acids, via an exchange reaction catalysed by transaminases. Therefore, if protein synthesis is occurring, ^{3}H will be incorporated into protein. When the tissue is returned to unlabelled medium, any ^{3}H incorporated into protein is stable but when protein degradation occurs, the ^{3}H of the amino

acid becomes available for exchange with H, and therefore any amino acids re-utilised for protein synthesis will not contain ^{3}H. Degradation rates can be measured by observing the amount of ^{3}H present in protein as a function of time.

However, as already pointed out the above methods were unsuitable for our studies as arginine: and leucine: tRNA ligases might be inactivated by the repeated and laborious purifications required to isolate the enzyme protein. Having established that both arginine: and leucine: tRNA ligases turn over, the question arises as to what might be the physiological significance of such protein turnover. As a general phenomenon it would seem a wasteful process, in terms of cellular economy, to degrade protein in fast growing cells such as tobacco XD cells in M-1D. In nitrateless M-1D, however, degradation would be the only way in which amino acids could be made available for protein synthesis. In micro-organisms (Goldberg et al. 1974) and mammalian cells (Goldberg & Dice, 1974), it is thought that degradation acts in a protective way by preventing accumulation of abnormal proteins which may have arisen by mistakes in synthesis, premature release of incomplete polypeptides, intracellular denaturation or chemical modification. The accumulation of such aberrant proteins would be especially serious to non growing cells such as those in higher organisms which, unlike bacteria, cannot dilute them out by rapid growth.

Degradation may also be an important factor in regulating enzyme activity. This is unlike the situation in growing bacteria, where there is little protein turnover and enzyme levels can be determined either by gene expression, i.e. either synthesis or no synthesis, or by diluting out during periods of rapid growth. In tobacco XD cells, however, regulation of arginine: and leucine:

tRNA ligases must be viewed against a background of both synthesis and degradation. Therefore, although arginine: and leucine: tRNA ligases are synthesised de novo control of degradation might be instrumental in regulating their level, and enzyme activity might be determined by a fine balance between synthesis and degradation. It might also be expected that for ordered changes in enzyme levels, there should be some sort of interaction between synthesis and degradation. In higher plants, however, little is known about the control of degradative processes and even less as to how synthesis and degradation might be integrated. Circumstantial evidence for the existence of specific protein proteases in plants comes from experiments performed with nitrate reductase, where inhibitors of protein synthesis, such as cycloheximide and actinomycin D, greatly retard the loss of nitrate reductase in induced barley plants placed in the dark (Travis et al., 1969). Similar observations have been made with phenylalanine ammonia lyase (Zucker, 1972), UDP-galactose polysaccharide transferase (Sussman & Sussman, 1965) and fatty acid synthetase enzymes (Willemot & Stumpf, 1967).

An insight into the possible mechanisms of degradation by proteolytic enzymes has come from the work of Katunuma (1975). It has been demonstrated that rat liver enzymes requiring vitamin B_6 as co-enzyme (pyridoxal enzymes) appear to be protected against proteolysis when bound to the co-enzyme. In the absence of co-enzyme this protection is lost due to a conformational change which renders the apo-enzyme more susceptible to proteolysis. It can therefore be envisaged that other enzymes can be made susceptible by undergoing similar conformational changes via interactions not only with co-enzymes, but also with ligands, by phosphorylation and adenylylation, or even by membrane interaction. Katanuma proposes that group specific proteases catalyse the degradation of

a susceptible enzyme via limited proteolysis. Subsequently, the products are broken down in a step-wise manner by other proteases. If proteolytic enzymes are important factors in the regulation of our enzymes, since proteases are themselves proteins, their activity must also be regulated in some way. They could themselves be turning over or be activated by other proteases, or their activity and specificity could be related to membrane binding. An insight into the complex mechanism that may be involved comes from an extracellulary excreted protease from Neurospora crassa which appears to be controlled by multiple regulatory circuits (Hanson & Marzluf, 1975).

In conclusion, we have shown that arginine: and leucine: tRNA ligases turn over in common with a number of other plant enzymes (Table 2), but the mechanisms which control both synthesis and degradation are unknown. The evidence points to some form of metabolic regulation in which the control of synthesis of these enzymes is related to the general status of the cell. However, this involves only enzyme synthesis and does not envisage a role for degradation.

We cannot say whether enzyme activity measured in our studies represents true in vivo activity. This is because low molecular weight compounds which could be involved in other regulatory mechanisms of these enzymes, such as allosteric activators, have been removed during partial purification of enzyme extracts by Sephadex chromatography, and in addition, enzyme activity may be altered simply by removing the enzymes from their cellular environment, or by experimental manipulation of the tissue during extraction or during subsequent assay. The only way to determine whether the enzyme activity measured in our studies represents the true in vivo situation would be to measure in vivo rates of aminoacylation, and although methods are available (Ehresmann, 1974), they involve long

and difficult procedures.

Three approaches could be used to extend the work in this thesis:-

(1) Arginine: and leucine: tRNA ligases could be highly purified using affinity chromatography techniques (Cuatrecasas, 1970; Jakubowski & Pawelkiewicz, 1973) and *in vitro* observations applied to the *in vivo* situation.

(2) The subcellular distribution of the multiple ligase species could be determined by using sucrose gradient techniques (Moore & Beevers, 1974).

(3) Further investigations could be made into the possibility that free amino acids, aminoacyl-tRNA or perhaps complexes between amino acid and tRNA or aminoacyl-tRNA and tRNA, are important in regulating these enzymes. In our studies, a role for amino acids in such a capacity could not be demonstrated. Exogenously supplied amino acids, however, may not be equivalent to endogenously synthesised amino acids. This problem could be overcome by isolating mutant cell lines which overproduce specific amino acids. Such cell lines have been isolated in carrot (Widholm, 1972a) and tobacco (Widholm, 1972b), where cells resistant to 5-methyl tryptophan overproduce tryptophan due to an altered anthranilate synthetase, which was no longer feed back inhibited by tryptophan to the same extent as the wild type cells.

If, however, aminoacyl-tRNA is involved in the regulatory process, it would be necessary to perturb *in vivo* levels of aminoacyl-tRNA and examine the effect on the cognate ligase. We looked for, but failed to find, a suitable amino acid analogue which would perturb *in vivo* levels of aminoacyl-tRNA either by competitively inhibiting a specific amino acid:tRNA ligase or by reducing the level of a specific amino acid. An alternative approach would be to isolate mutant cells with altered amino acid:

tRNA ligases. This technique has been successfully used in micro-organisms, where cell lines resistant to amino acid analogues have been found to possess amino acid:tRNA ligases with a lowered affinity for the analogue and therefore reduced production of the cognate aminoacyl-tRNA. Such cell lines might also have to be supplemented with amino acid in order to grow at the normal wild type rate. This type of mutant would therefore be a suitable tool not only for examining the role of aminoacyl-tRNA in regulating amino acid:tRNA ligases but also for investigating the stringent response in higher plants, and the role of aminoacyl-tRNA in regulating amino acid biosynthesis. Of similar value would be the isolation of mutants which are auxotrophic for a specific amino acid. However, since mutations conferring auxotrophy are recessive, their isolation needs to be carried out on haploid material. The advent of anther and pollen culture techniques has made this feasible and suggests that it may soon be possible to widely apply the techniques of biochemical genetics to higher plants.

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